Non-genomic and Genomic Effects of Estrogen and Progesterone on Mammalian Arteries

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Declaration

The experiments reported in this dissertation were carried out in the Department of Physiology, the Chinese University of Hong Kong, between August 1999 and June 2001. The work is solely that of the author. No part of this dissertation is being concurrently submitted for any other degree, diploma or other qualification at this or any other institutions.

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Abbreviations

AC	Adenylate cyclase
Ach	Acetylcholine
ATP	Adenosine triphosphate
BK_{Ca}	Large conductance Ca^{2+}-activated K^+ channel
$[Ca^{2+}]_i$	Intracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
ENOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
EREs	Estrogen response elements
ERT	Estrogen replacement therapy
FSH	Follicle stimulating hormone
GC	Guanylate cyclase
GTP	Guanosine triphosphate
HDL	High-density lipoprotein
IBMX	3-Isobutyl-1-methylxathine
Iso	Isoproterenol
K_{ATP} channel	ATP-sensitive K^+ channel
L-arg	L-arginine
LDL	Low-density lipoprotein
LH	Luteinizing hormone
L-NAME	N^G-nitro-L-arginine methyl ester
MAPK	mitogen-activated protein kinases
MB	methylene blue
MEK	MAPK kinases
NO	Nitric oxide
NOS	Nitric oxide synthase
PDA	Phorbol 12,13-diacetate
Phe	Phenylephrine
Rp-cAMPS	Rp-cAMPS triethylamine

Rp-8-cGMP	Rp-8-pCRT-cGMPS triethylamine
SHBG	Sex hormone-binding globulin
SMC	Smooth muscle cell
U46619	9,11-dideoxy-11$_\alpha$9$_\alpha$-epoxymethanoprostaglandin F2$_\alpha$

Abstract

The beneficial effects of estrogen include lowering of low-density lipoprotein cholesterol and decreased vascular contractility. However, the precise pathways, and the cellular mechanisms by which female sex steroid hormones influence the activity of various vasculatures are incompletely understood. An increasing amount of evidence suggests that nitric oxide production, modulation of the vascular responsiveness to endogenous vasoconstrictors or vasodilators may play an important role in the vascular effects of female sex steroid hormones.

I specifically proposed the following hypotheses to be examined. (1) Endothelial nitric oxide may play a differential role in relaxation of arteries of different diameters induced by estrogen and progesterone. (2) β-Adrenoceptor agonists and 17β-estradiol may exert mutual synergistic effects on vessel tone. (3) Acute exposure to physiological level of 17β-estradiol may modulate β-adrenoceptor-mediated vasorelaxation. (4) Chronic treatment with 17β-estradiol may alter the vascular contractility. To this end, the experiments were carried out on several types of isolated rat arteries (*e.g.* aorta, mesenteric, and carotid artery) and on porcine coronary artery.

I have demonstrated that 17β-estradiol induced both endothelium-dependent and –independent relaxation in rat aortic rings, but only endothelium-independent relaxation in rat mesenteric arteries. In contrast, progesterone induced both endothelium-dependent and –independent relaxation in mesenteric arteries, but only endothelium-independent relaxation in rat aortas. N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM) and methylene blue (3-10 μM) attenuated relaxation induced by 17β-estradiol in aortas or to progesterone in mesenteric arteries. L-arginine (1 mM) significantly antagonized the effect of L-NAME on sex hormones-induced relaxation.

I have provided novel evidence for the synergistic interaction between β-adrenoceptor agonists and 17β-estradiol. 17β-Estradiol-induced relaxation was enhanced by 1-hr pretreatment with 1-3 nM isoproterenol, a β-adrenoceptor agonist. This effect was absent in endothelium-denuded mesenteric artery rings. Propranolol (3 μM), a β-adrenoceptor antagonist reduced the effect of isoproterenol (1 nM). ICI 118, 551, a β_2-adrenoceptor antagonist abolished the effect of isoproterenol. In contrast, atenolol, a β_1-adrenoceptor antagonist had no effect. Besides, fenoterol, a β_2-adrenoceptor agonist, also potentiated 17β-estradiol-induced relaxation. Incubation of endothelium-intact arteries with L-NAME and Rp-cAMPS triethylamine, an inhibitor of cAMP-dependent protein kinase, abolished isoproterenol enhancement of 17β-estradiol-induced relaxation. Rp8-pCRT-cGMPS triethylamine, an inhibitor of cGMP-dependent protein kinase Iα, significantly attenuated the effect of isoproterenol.

Short-term (20 minutes) or longer (1 hr) exposure to 17β-estradiol (0.1-1 nM) did not modify isoproterenol- and dobutamine-induced relaxation in most concentrations of β-adrenoceptor agonists tested. However, 1-hr exposure to 0.3 nM 17β-estradiol significantly potentiated relaxation produced by fenoterol in porcine coronary circumflex arteries. Tamoxifen (10 μM) antagonized this effect.

To study the chronic effect of 17β-estradiol, the rats were ovariectomized and divided into four groups, *e.g.* the ovariectomized, the ovariectomized plus estrogen replacement, the ovariectomized plus tamoxifen replacement, and the ovariectomized plus 17β-estradiol and tamoxifen replacement groups. I have clearly revealed a marked change in vascular contractility following ovariectomy. In the endothelium-denuded rat carotid arteries, the contractile responses to phenylephrine, U46619, or high K^+ were significantly enhanced in the ovariectomized rats. This enhancing effect

was completely reversed by estrogen therapy or was absent in endothelium-intact rings. Chronic treatment with tamoxifen (implanted pellet) had a similar effect to 17β-estradiol pellet. In contrast, acetylcholine-mediated relaxation was unaffected.

In summary, I have provided some novel findings concerning the arterial effects of female steroid hormones. Firstly, endothelial nitric oxide plays different role in relaxant response to 17β-estradiol and progesterone in the conduit vessel (aorta) and smaller-sized vessels (mesenteric artery). Endothelial nitric oxide contributes largely to endothelium-dependent relaxation induced by 17β-estradiol in the aortas or by progesterone in the mesenteric arteries. Secondly, 17β-estradiol-induced relaxation in mesenteric arteries was enhanced by isoproterenol via β_2-adrenoceptor activation. L-NAME or Rp-8-cGMPS eliminated or markedly inhibited the effect of isoproterenol, suggesting that isoproterenol may act on the endothelial cells to release nitric oxide and cGMP-dependent pathway was involved. A cAMP-dependent mechanism in endothelium was also involved in the effect of isoproterenol. These new findings suggest that β_2-adrenergic agonist and 17β-estradiol can synergies each other via several intracellular second messengers primarily in endothelium. Thirdly, both 17β-estradiol and progesterone concentration-dependently reduced the contractile response to the activator of protein kinase C, indicating an additional cellular mechanism by which steroid hormones reduce vessel tone evoked by PKC-dependent constrictors such as phenylephrine, endothelin and angiotensin II. Fourthly, acute exposure to physiological level of 17β-estradiol enhanced β_2-adrenoceptor-mediated coronary relaxation. Finally, ovariectomy and chronic 17β-estradiol altered the vascular reactivity mainly in endothelium-denuded arteries. These data suggest that chronic treatment of 17β-estradiol may modulate the properties of smooth muscle but not of endothelial cells.

Abstract in Chinese (簡述)

雌激素所帶來的好處包括降低低密度脂蛋白膽固醇 (low-density lipoprotein cholesterol) 和減低血管的收縮性。但是在細胞的水平上雌激素所致的血管舒張機理並不完全清楚。很多證據顯示女性荷爾蒙能增加一氧化氮合成及調節體內引致血管收縮物質和舒張物質。

因此我根據以下假設作爲研究目標：(1)由血管內皮細胞釋放出來的一氧化氮在女性荷爾蒙引起之血管舒張有著重要地位，但在大小不同的血管上有不同的重要性。(2) β腎上腺能受體激動劑 (β-adrenoceptor agonist) 與 17β-雌二醇 (17β-estradiol) 有相互作用。 (3) 生理濃度的 17β-雌二醇能影響 β腎上腺能受體所引致的血管舒張。(4) 長時期預處理 17β-雌二醇能影響血管之收縮反應。本實驗採用離體大白鼠之血管 (包括：大動脈；腸系膜上動脈和頸動脈) 和豬心臟血管，以測量 17β-雌二醇和黃體酮引起之張力變化。

本研究發現 17β-雌二醇在大白鼠的大動脈內能引起內皮細胞依賴性 (endothelium-dependent) 和內皮細胞非依賴性(endothelium-independent) 之血管舒張，但只能在腸系膜上動脈引起內皮細胞非依賴性之血管舒張。相反黃體酮在腸系膜上動脈能引起內皮細胞依賴性和內皮細胞非依賴性之血管舒張，但只能在大動脈引起內皮細胞非依賴性之血管舒張。預處理一氧化氮合成酶抑壓制 N^G-nitro-L-arginine methyl ester (L-NAME) 和 guanlyate cyclase 抑制劑 methylene blue 都可抑制 17β-雌二醇和黃體酮引起之血管舒張。而 L-arginine 則能拮抗 L-NAME 之作用。

在內皮完整的血管上，預處理低濃度 (1-3 nM) 的異丙腎上腺素 (isoproterenol, 非特異性 β腎上腺能受體激動劑) 能有效地提升由 17β-雌二醇引

致的血管舒張。去除血管內皮細胞能抑制異丙腎上腺素的作用。若在使用異丙腎上腺素之前，先加進 propranolol (非特異性β腎上腺能受體抑壓劑) 或 ICI 118,551 (β_2腎上腺能受體抑壓劑)都可抑制雌二醇引致作用。但是 atenolol (β_1腎上腺能受體抑壓劑)則沒有作用。另外，fenoterol (β_2 腎上腺能受體激動劑)也有助雌二醇引致的血管舒張。另一方面，在內皮細胞完整的血管，預處理 L-NAME 、Rp-cAMPS (依賴環腺苷酸蛋白激酶抑制劑) 或 Rp-8-pCRT-cGMPS triethylamine (依賴環鳥苷酸蛋白激酶Iα抑制劑) 都能有效地削弱異丙腎上腺素提高雌二醇所引致的血管舒張作用。

另外，本研究發現無論短期或長期預處理 17β-雌二醇都不能影響 isoproterenol 和 dobutamine (β_1 腎上腺能受體激動劑) 所引致的豬冠狀動脈血管舒張。可是經過一小時預處理 0.3nM 17β-雌二醇能有效地提升 fenoterol 所引致的血管舒張，而 tamoxifen 則能拮抗 17β-雌二醇之作用。

最後一項實驗發現雌性大白鼠在接受 ovariectomy (切除卵巢手術) 後，其血管的收縮性大有不同。在血管內皮細胞缺失的頸動脈上，ovariectomy 能大大提升 phenylephrine, U46619 和高濃度外鉀離子所引致的血管收縮。在接受雌激素療法後，此情況則回復正常。

研究結果顯示：(1) 在內皮完整的血管上，17β-雌二醇引起的大動脈血管舒張和黃體酮引起之腸系膜上動脈血管舒張是跟隨內皮細胞的一氧化氮機制；(2) 異丙腎上腺素增強 17β-雌二醇引致的血管舒張，主要是透過激活 β_2-腎上腺能受體機制。另外，預處理一氧化氮抑制劑，例如：L-NAME 或 Rp-8-cGMPS 都能有效地削弱異丙腎上腺素之功能，顯示出其作用是透過刺激內皮細胞以產生一氧化氮和環鳥苷酸系統機制。這些新發現顯示 β_2腎上腺能受體激動劑和 17β-雌

二醇之相互作用是透過幾種內皮細胞的第二信使(second messenger) 來傳遞訊息；(3) 17-β雌二醇和黃體酮都能以濃度依賴性地抑壓蛋白激酶C 激活劑所致的血管收縮，並相信女性激素也可減低其他透過蛋白激酶 C 系統機制的血管收縮物質，例如：正腎上腺素，內皮素和血管緊張肽素 II 。(4) 短期預處理 17β-雌二醇能提升 β_2 腎上腺能受體控制之血管舒張。(5) 把 17β-雌二醇植入已接受切除卵巢手術的大白鼠能回復內皮缺失之血管的收縮性，顯示長期預處理 17β-雌二醇能影響血管平滑肌的特性。

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Chapter 1 Introduction

1.1. Steroid Hormones

Hormones can be divided into three groups on the basis of chemical structure: (1) amino acid-related hormones, (2) peptides and protein hormone, and (3) steroid hormones. Their chemical nature, such as, where it is derived, how it is carried in the blood and its cellular mechanism of action are different.

In mammalian systems, there are seven groups of steroid hormones that can be classified on both a structural and biological basis. They are (1) androgens, (2) estrogens, (3) glucocorticoids, (4) mineralocorticoids, (5) progestagens, (6) vitamin D steroids, and (7) bile acids (Norman & Litwack, 1997).

All steroid hormones are lipids that are derived from cholesterol. Except vitamin D steroids, the basic structure of all other steroids has a cyclopentanoperhydrophenanthrene ring as their core, which is shown in Figure 1a. Since it is derived from cholesterol and synthesized on smooth endoplasmic reticulum, steroid hormones are non-polar and fat-soluble. Due to these properties, once steroid hormones are produced, they simply diffuse across the plasma membrane of the steroid-producing cell and enter the interstitial fluid and then the blood. Therefore, glandular storage of steroids is minimal. They are not readily soluble in blood and hence are transported bound to proteins. Furthermore, these compounds are absorbed fairly in the gastrointestinal tract and therefore they can be administered orally.

1.1.1. Synthesis of estrogens and progesterone

The estrogens are a group of hormones, which include estrone (E_1), estradiol (E_2) and estriol (E_3). At least six different estrogens have been isolated from the plasma of

human females (Tortora and Grabowski, 1996). However, only three are present in significant quantities: estrone, 17β-estradiol and estriol.

In non-pregnant females, estrogens are produced in follicular cells of ovaries under the control of gonadotrophin from the anterior pituitary, follicle stimulating hormone (FSH). FSH stimulates the initial secretion of estrogens by converting the cholesterol to pregnenolone, which is then converted to androstenedione or testosterone, in some cases via progesterone. Testosterone is then converted to 17β-estradiol by aromatase enzymes. Few days before ovulation, another gonadotrophin, luteinizing hormone (LH) stimulates the further development of ovarian follicles and their full secretion of estrogen brings about ovulation and promotes formation of the corpus luteum. On the other hand, adipose tissue also expresses aromatase enzymes and is therefore able to convert androgens to estrogens. In males and postmenopausal females adipose tissue is the major source of estrogen.

Meanwhile, the mature follicles produce a small amount of progesterone several days before ovulation. After ovulation, the major source of progesterone is the cells of corpus luteum. It is derived from cholesterol via pregnenolone under the action of 3β-steroid dehydrogenase (Norman & Litwack, 1997). The function of progesterone in this phase is to synergies with estrogens to prepare the endometrium for implantation of a fertilized ovum. Moreover, high levels of progesterone may give negative feedback to hypothalamus and anterior pituitary to inhibit secretion of gonadotropin releasing hormone and LH.

In this study, I focused on examining the vascular effects of two steroids: 17β-estradiol and progesterone. The chemical structures of these two steroids are shown in Figure 1b and 1c. The synthetic route for the conversion of cholesterol to estrogens and progesterone is shown in Figure 2. These two steroids have commonly been used in contraception (Pharmaceutical Society of Australia, 1998) and hormone

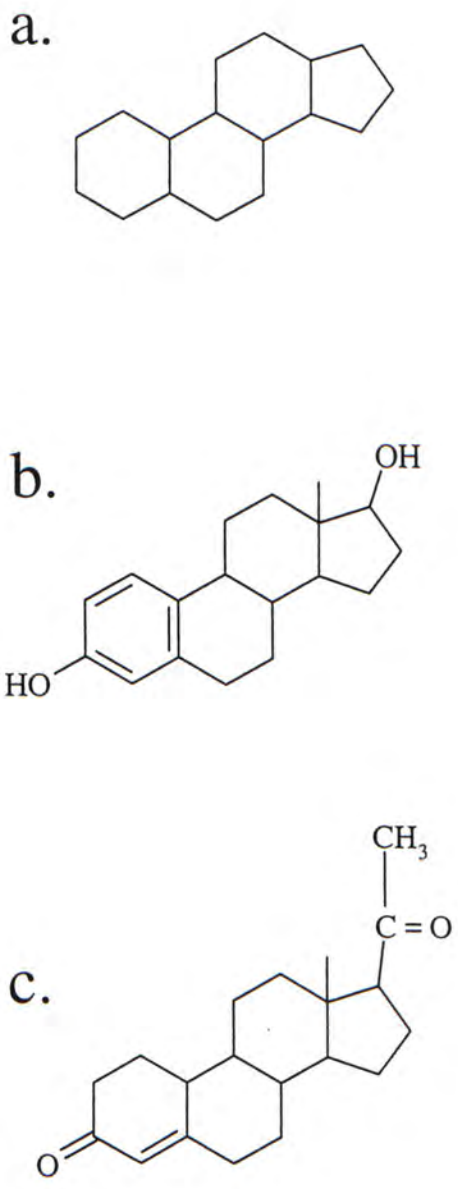

Figure 1

The chemical structures of (**a**) cyclopentanoperhydrophenanthrene, (**b**) 17β-estradiol and (**c**) progesterone.

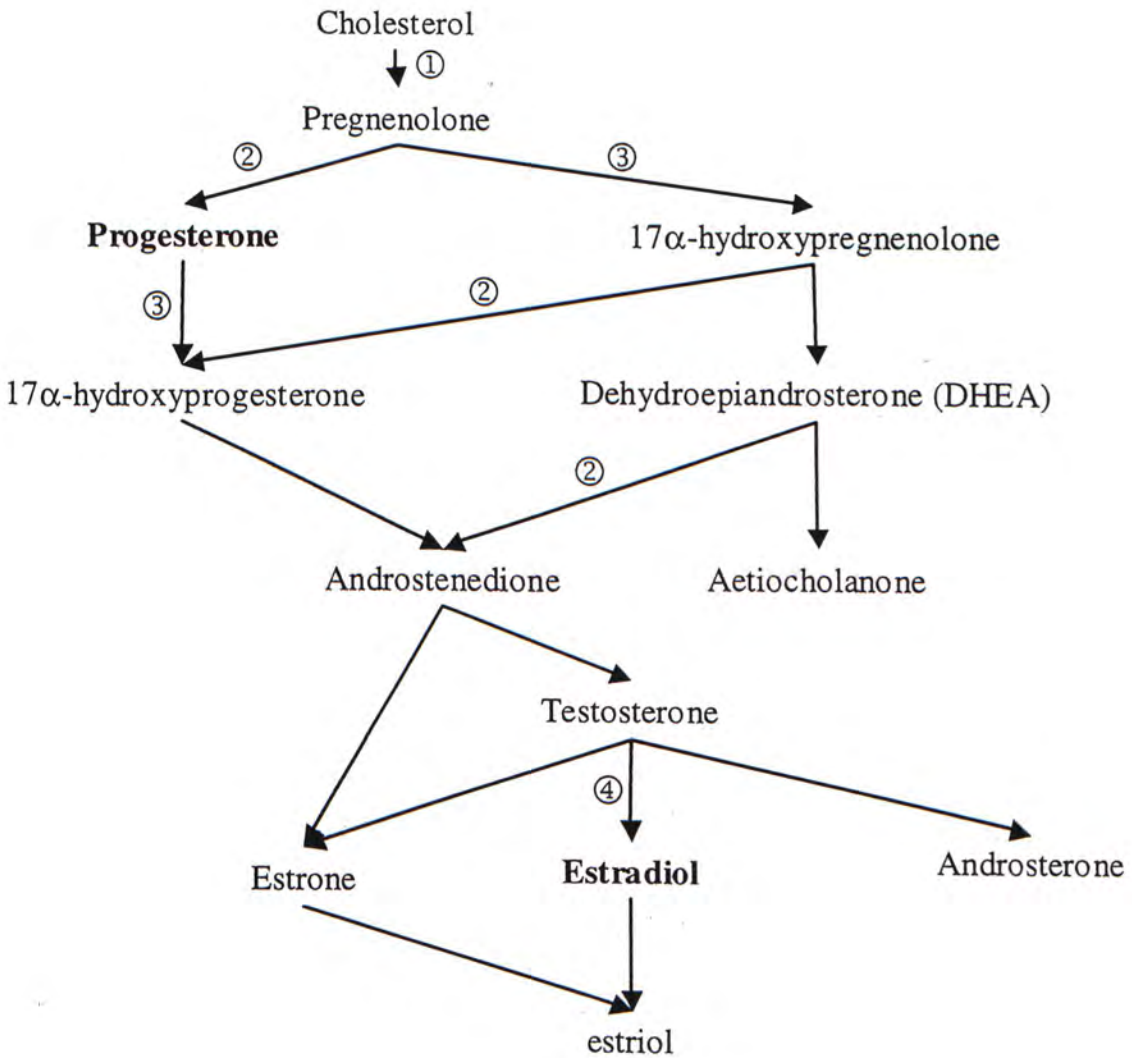

Figure 2

The synthetic route for the conversion of cholesterone to estrogens and progesterone by the ovaries. The circled numbers represent the key enzymes involved in the process. ① desmolase; ② 3β-steroid dehydrogenase; ③ 17α-hydroxylase; ④ aromatase.

replacement therapy in postmenopausal women for several decades (Stampfer *et al.*, 1991; Grady *et al.*, 1992; Godsland, 2001).

1.2. Cellular Mechanisms of Female Steroid Hormones

Steroids can act through two basic mechanisms: genomic and non-genomic. The classical genomic action is mediated by specific intracellular receptors, whereas the primary target for the non-genomic one is the cell plasma membrane. In the following, estrogen is taken as an example to explain these two mechanisms in cardiovascular system.

1.2.1. Genomic actions of female steroid hormones

Once steroid hormone particles are combined with sex hormone-binding globulin (SHBG) after synthesis, they are carried to the target cells. In cardiovascular system, estrogen receptor was found in both endothelium and vascular smooth muscle of blood vessels (Horwitz and Horwitz, 1982). After chronic exposure of estrogen, it binds to the intracellular receptors. The estrogen-receptor complex then binds to specific DNA sequences and estrogen response elements (EREs), resulting in altered transcription of specific mRNA and subsequent protein synthesis. For example, prolonged estrogen treatment increases mRNA for endothelial nitric oxide synthase (NOS), which suggests that increased NOS activity results from enzyme induction and an increase in protein synthesis (Goetz *et al.*, 1994; MacRitchie *et al.*, 1997). This elevation of NOS may increase production of nitric oxide (NO), which then diffuses to the underlying vascular smooth muscle. In addition, smooth muscle NOS may also be increased by estrogens, explaining the genomic modulation of endothelium-denuded vascular tone that has recently been observed (Binko *et al.*, 1998; Binko & Majewski, 1998) (Figure 3).

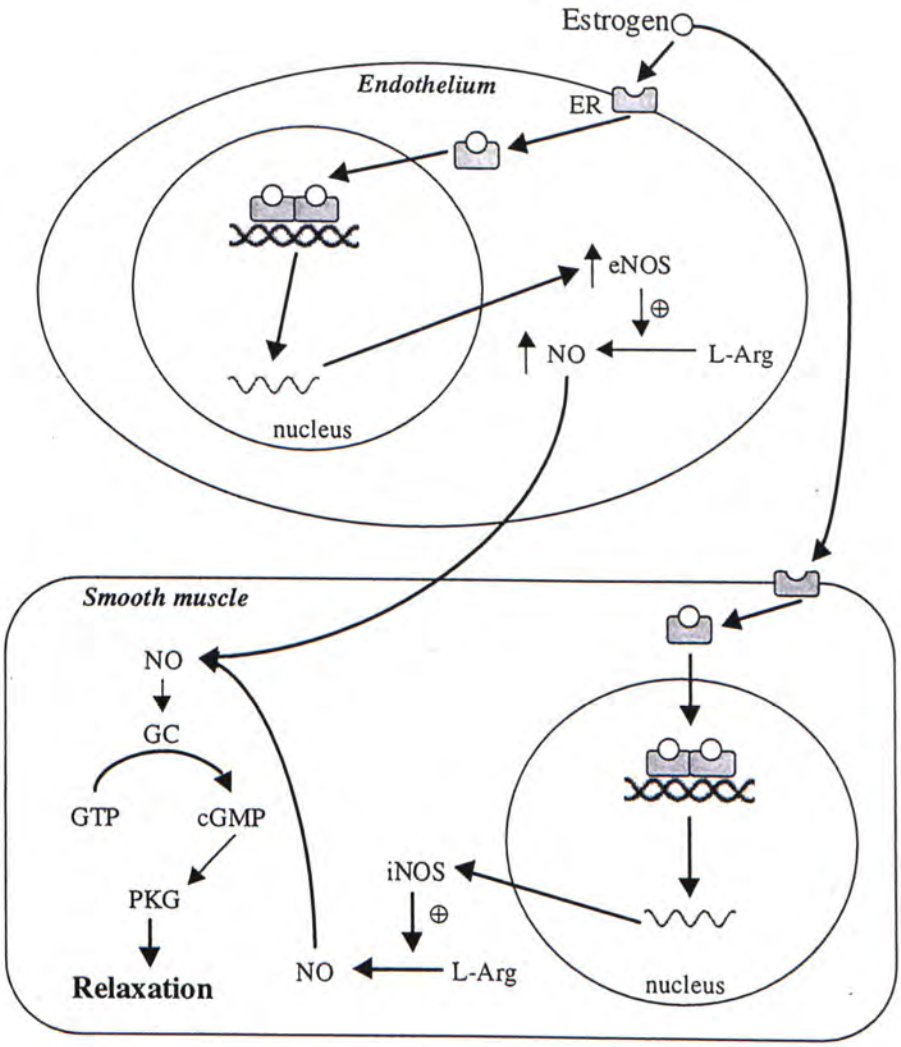

Figure 3

The proposed genomic mechanisms by which vessel tone may be altered following chronic estrogen exposure. These involve an increased synthesis of endothelial NOS and release of NO from endothelium. Moreover, inducible NOS in smooth muscle may also be increased by estrogen, explaining the genomic modulation of endothelium-denuded vascular tone (Binko *et al.*, 1998; Binko & Majewski, 1998). cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; GC, guanylate cyclase; GTP, guanosine triphosphate; iNOS, inducible nitric oxide synthase; L-Arg, L-arginine; PKG, cGMP-dependent protein kinase.

1.2.2. Non-genomic actions of female steroid hormones

In contrast to genomic steroid action, non-genomic steroid effects are principally characterized by their insensitivity to inhibitors of transcription and translation (actinomycin D and cycloheximide). For example, it is reported that inhibitors of protein and RNA synthesis have no effect on the acute endothelium-independent vasodilatory effects of estrogens in a number of different vessels (Kitazawa *et al.*, 1997; Shaw *et al.*, 2000). This rapid and acute responses generally appear to be endothelium-independent, which has been shown in a wide range of arteries, such as rat aorta (Andersen *et al.*, 1999), rat mesenteric and coronary arteries (Shaw *et al.*, 2000) and rabbit coronary arteries (Jiang *et al.*, 1991). However, there is evidence that acute exposure to estrogen may also alter the eNOS activity. It has been described that acute application of 17β-estradiol increases the eNOS activity via a Ca^{2+}-dependent mechanism (Shaul, 1999) (Figure 4).

On the other hand, a number of studies reported that progesterone also exerts a non-genomic action. For example, progesterone at micromolar concentrations induced concentration-dependent relaxations of the isolated rat saphenous artery rings within 10 minutes (Kakusc *et al.*, 1998). Moreover, progesterone caused coronary relaxation by inhibiting Ca^{2+} entry through voltage-gated Ca^{2+} channels in coronary smooth muscle. Besides, 17β-estradiol and testosterone have a similar action (Crews & Khalil, 1999).

1.2.3. Estrogen antagonists

1.2.3.1 Classification of estrogen antagonists

Steroid antagonists can be either steroidal analogues or nonsteroidal compounds that function as selective inhibitors of steroid hormone action. According to MacGregor

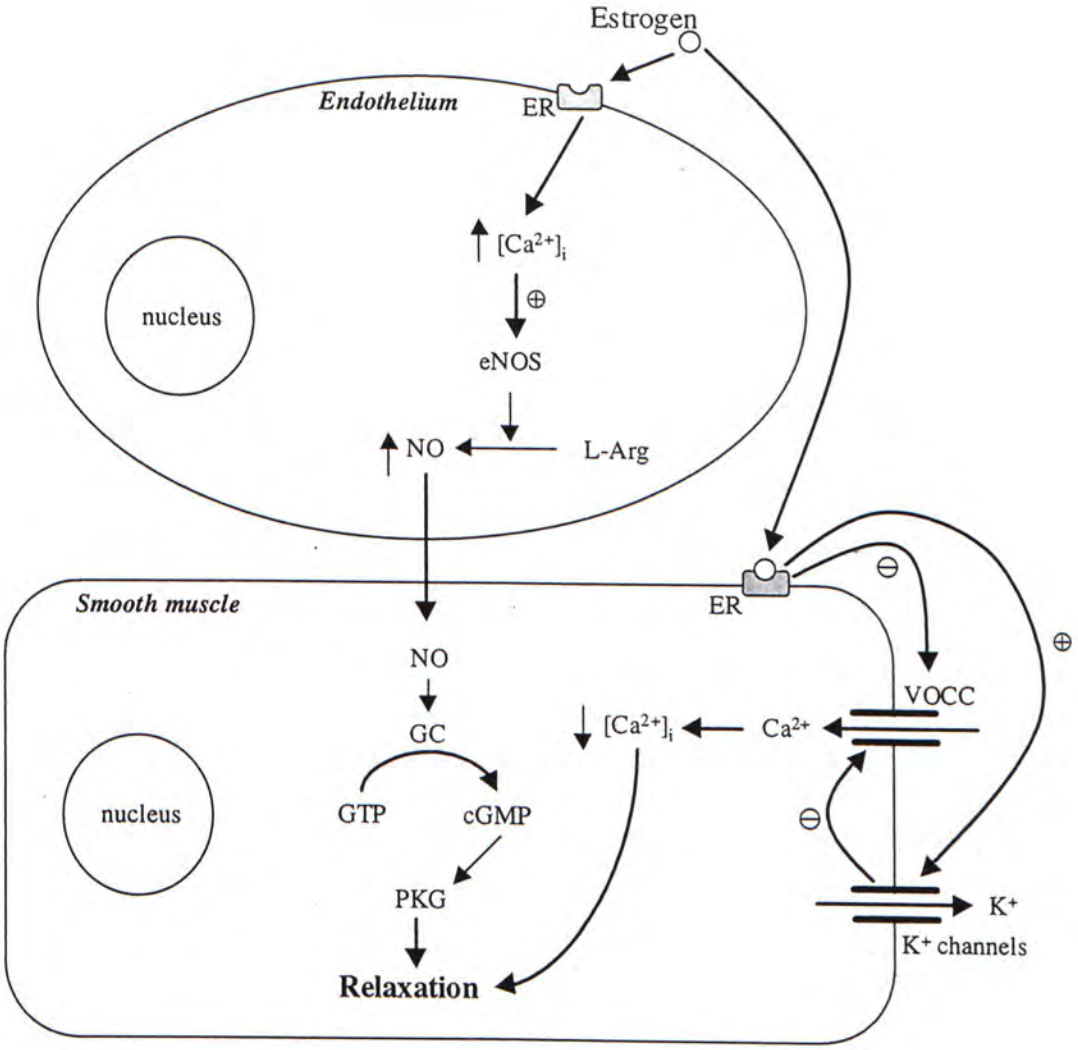

Figure 4

The proposed non-genomic mechanisms underlying the acute vasodilator effect of estrogen. These involve (1) Stimulation of NO release via increased $[Ca^{2+}]_i$ in endothelial cells. (2) Inhibition of voltage-operated Ca^{2+} channels (VOCC) and thus a decrease in $[Ca^{2+}]_i$ in vascular smooth muscle. (3) Activation of K^+ channels, membrane hyperpolarization and subsequent inhibition of Ca^{2+} influx through VOCC in vascular smooth muscle. cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; GC, guanylate cyclase; GTP, guanosine triphosphate; L-Arg, L-arginine; NO, nitric oxide; PKG, cGMP-dependent protein kinase; VOCC, voltage-operated Ca^{2+} channels.

and Jordan, estrogen antagonists are divided into two major groups: type I and II (MacGregor & Jordan, 1998).

Type I estrogen antagonists include those compounds that exhibit mixed estrogenic and anti-estrogenic actions. These compounds are also called selective estrogen receptor modulators (SERMs) (Sato *et al.*, 1994). SERMs are structurally diverse non-steroidal agents that bind to both estrogen receptor α (ERα) and β (ERβ) receptors, and produce estrogen agonistic effects in some tissues and estrogen antagonistic effects in others (Sato *et al.*, 1994). The action of these compounds is thought to be tissue- and species-specific. For example, tamoxifen is anti-estrogenic in breast tissues (Jordan & Morrow, 1999), but it causes endometrial stimulation and increases the likelihood of endometrial cancer (Barakat, 1996; Barakat, 1998). Due to this characteristic, SERMs are being evaluated for a number of estrogen-related diseases, including hormone-related cancers, postmenopausal osteoporosis and cardiovascular disease. The most commonly used drugs that exhibit a SERM property for clinical use are tamoxifen, raloxifene, clomiphene and toremifene.

Type II estrogen antagonists are free of estrogen-like properties in laboratory assays. These compounds can be considered as pure anti-estrogenic agents because their action is not tissue- and species-specific. They always exert antagonistic effects in all estrogen-targeted tissues, such as the mammary tissue, uterus and bones (Gradishar and Jordan, 1997). The most commonly used drugs of this group are ICI 182,780 and ICI 164,384. Table 1 tabulates the compounds falling into these two categories.

1.2.3.2. Mechanisms of estrogen antagonists.

Type I antagonists are competitive inhibitors of the binding of estradiol to estrogen receptor. For example, raloxifene binds to estrogen receptor, which retains partial transcription activity (Brzozowski *et al.*, 1997). However, type II antagonists bind to

estrogen receptor to prevent the complex binding to EREs (Fawell *et al.*, 1990).

Table 1 *Classification of estrogen antagonists*

Type I (SERMs)	Type II (Pure anti-estrogen)
Tamoxifen	ICI 182,780
Raloxifen	ICI 164,384
Clomiphene	RU 58688
Toremifene	EM-800
	EM-139

1.3. Chronic (genomic) Effects of 17β-Estradiol and Progesterone

1.3.1. Effects on lipid metabolism

Estrogens protect against the development and progression of atherosclerosis (Walsh *et al.*, 2000). The best known beneficial effect of estrogens on coronary heart disease (CHD) risk is the lipid hypothesis. It was first reported in 1987 that estrogens affected the lipoprotein profile by increasing high-density lipoprotein (HDL) cholesterol levels and decreasing low-density lipoprotein (LDL) cholesterol level (Bush *et al.*, 1987).

Recent studies have revealed that estradiol acts separately on the LDL particle and the artery wall to reduce LDL accumulation (Walsh *et al.*, 2000). It was found that estradiol protects LDL from modification and subsequent accumulation due to its antioxidant property (Walsh *et al.*, 2000), although the underlying mechanism is still unclear. Many biomedical and cell culture studies demonstrate that estrogen acts to

scavenge oxygen radicals in order to protect LDL from oxidative modification (Mooradian, 1993; Rifici and Khachadurian, 1992). However, some other studies suggest that estradiol within physiological concentrations may not function as an antioxidant (Santanam *et al.*, 1998).

Moreover, estradiol decreases basal LDL accumulation by decreasing arterial permeability in rats (Walsh *et al.*, 2000). Paradoxically, other investigators found that estrogen did not affect aortic permeability to LDL in rabbits (Haarbo *et al.*, 1994), indicating a species difference.

On the other hand, progesterone has a similar function to estrogen on lipid metabolism. It was reported that treatment of postmenopausal women with levonorgestrel (a kind of progesterone) resulted in a reduction of the serum concentration of HDL_2 by 20 %. Moreover, the concentrations of total triglycerides, total phospholipids, HDL, HDL_2, HDL-phospholipids and apolipoprotein A1 were decreased by 10 to 30 % (Kuusi *et al.*, 1993; Kauppinen-Makelin *et al.*, 1992).

1.3.2. Effects on cell proliferation

The protective effect of estrogen in animals against vascular injury is partly mediated by its anti-proliferative effect on vascular smooth muscle cells. Smooth muscle cells (SMC) proliferation is an important process after blood vessels were injured (Ross and Glomset, 1973; Clowes *et al.*, 1983). In 1977, it was reported that estrogen suppressed surgically induced vascular hyperplasia in the rabbit aorta (Rhee *et al.*, 1977). Later on, more experiments provided further evidence for the inhibitory effect of estrogen on SMC proliferation. Myointimal thickening after mechanical or lipid-induced damage of the vessel wall can be attenuated markedly following 17β-estradiol treatment (Nishigaki *et al.*, 1995). In cell culture experiments, it was found that 17β-estradiol inhibited proliferation of rabbit aortic SMC stimulated with

hyperlipedemic serum (Fisher-Dzoga *et al.*, 1983). Another set of experiments showed similar results in cultured human vascular SMC (Morey *et al.*, 1997), in which estrogen inhibited the mitogen-activated protein kinases (MAPK) and MAPK kinases (MEK) activity and stimulated *c-fos* and *c-myc* expression. These findings suggest that estrogen can suppress growth factor-induced vascular SMC proliferation and thus prevents thickening of the vascular wall.

1.3.3. Effects on endothelial cells

Estrogen does not only target on SMC, but also exerts potent biological effects on the endothelial cells. *In vivo* studies, estrogen can accelerate re-endothelialization in carotid arteries of ovariectomized rat, which was implanted estradiol-pellets after arterial injury (Krasinski *et al.*, 1997). Later on, supplementary data have indicated that this process is likely mediated through estrogen receptor-α but not estrogen receptor-β (Brouchet *et al.*, 2001).

Morales *et al.* reported that estrogen had a potent effect on endothelial cell migration and proliferation (Morales *et al.*, 1995). Using either a Boyden chamber assay or scratching a monolayer of endothelial cells, three-fold increases in cell migration were observed. In addition, estrogen-treated cells migrated into the wound three times faster than untreated cells after a confluent monolayer of cells was wounded by scraping (Morales *et al.*, 1995). Estrogen receptor antagonist ICI 182,780 inhibited the effect of estrogen. This process is important in the angiogenesis, because new blood vessel formation involves migration of the endothelial cells (Folkman, 1995).

1.4. Acute Effects of 17β-Estradiol and Progesterone

1.4.1. Role of endothelium in 17β-estradiol or progesterone relaxation

Estrogen receptors have been identified in both endothelium and the underlying vascular smooth muscle and they are thus physiological targets for estrogen action (Orima *et al.*, 1995). Nitric oxide and other endothelium-derived vasoactive factors are potent vasodilators which release is stimulated by many endogenously occurring substances. 17β-Estradiol mediated endothelium-dependent vasodilatation was studied in the past. 17β-Estradiol reduced vasoconstriction in the endothelium-denuded rat aortas probably through inducible production of nitric oxide (Binko & Majeweki, 1998). It has been reported that an enhanced vasodilator response in female rat aorta was abolished by endothelium removal (McNeill *et al.*, 1996). After long-term treatment of estrogen, aortic rings with intact endothelium were super-sensitive to noradrenaline, but such results did not occur in endothelium-denude rings (Moura & Marcondes, 2001). However, conflicting results have also been reported regarding the role of endothelium in vasodilatation induced by female sex steroid hormones. For example, nitric oxide was partly involved in the estrogen-induced relaxation in the rabbit aorta (Ma *et al.*, 1997), but not in the rabbit coronary arteries (Jiang *et al.*, 1991). Preliminary data from our laboratory demonstrate that nitric oxide contributes largely to the endothelium-dependent relaxation induced by 17β-estradiol in the rat aortas but not in the rat mesenteric arteries (Chan *et al.*, 2001a).

On the other hand, the role of endothelium in progesterone relaxation remains unclear. Our results showed that progesterone caused vasorelaxation in rat endothelium-intact mesenteric arteries but not in endothelium-denuded rings (Chan *et al.*, 2001a). However, progesterone was found to induce only endothelium-independent

relaxation in rabbit coronary arteries (Jiang *et al.*, 1992b). The cause of the difference between results obtained from two research groups is not known but it may be related to difference in experimental animal species.

1.4.2. Involvement of plasma membrane estrogen receptors

In 1977, evidence was provided to show that a plasma membrane estrogen binding protein rapidly responded to estradiol (Pietras & Szego, 1977). Since then, many functional studies on the rapid, non-genomic effects of 17β-estradiol have supported for the existence of the membrane estrogen receptor. Estrogen can trigger intracellular Ca^{2+} spikes in endothelial cells (Rubio-Gayosso *et al.*, 2000), increases Ca^{2+} efflux in vascular smooth muscle cells (Prakash *et al.*, 1999), stimulates adenylate cyclase (Aronica *et al.*, 1994) in a few seconds to minutes. The membrane estrogen receptor might also be involved in regulation of transcription, considered being the primary function of the nuclear estrogen receptor (Halachmi *et al.*, 1994).

At the same time, membranous binding of fluorescently labeled membrane-impermeant estrogen has been demonstrated in endothelial cells (Pyo *et al.*, 1999) and in pituitary tumor cells (Pappas *et al.*, 1995), which further supports for the presence of plasma membrane estrogen receptor.

1.4.3. Role of Ca^{2+} channel and K^+ channel in estrogen relaxation

As mentioned in Section 1.4.2., estrogen modulates Ca^{2+} influx through membrane estrogen receptor. In smooth muscle cells, acute administration of 17β-estradiol produced inhibition of L-type voltage-operated Ca^{2+} currents recorded with patch-clamp technique (Nakajima *et al.*, 1995), and a decrease in intracellular calcium concentration ($[Ca^{2+}]_i$) (Shan *et al.*, 1994). 17β-Estradiol also inhibited BAY K 8644 (a dihydropyridine derivative which opens voltage-dependent Ca^{2+}

channels)-induced elevation of $[Ca^{2+}]_i$ (Prakash *et al.*, 1999). In contrast, estrogens have no effect on Ca^{2+} release from intracellular stores in vascular smooth muscle (Kitazawa *et al.*, 1997). Therefore, it is concluded that the acute endothelium-independent vasodilatory response to estrogen is partly due to inhibition of Ca^{2+} influx across the plasma membrane of vascular SMC.

Apart from inhibition of the Ca^{2+} channels, activation of K^+ channels may also contribute to the estrogen-mediated vasorelaxation. 17β-Estradiol was shown to activate large conductance Ca^{2+}-activated (BK_{Ca}) and voltage-activated K^+ channels through a cGMP-dependent mechanism, thus indirectly reducing Ca^{2+} influx (White *et al.*, 1995). However, 17α-estradiol had no effect.

On the other hand, the effect of estrogen on Ca^{2+} influx in other cell types is opposite to that in smooth muscle cells. It has been shown that 17β-estradiol induced a fast increase in $[Ca^{2+}]_i$ in rat osteoblast (Lieberherr *et al.*, 1993), hepatocytes (Sanchez-Bueno *et al.*, 1991), and vascular endothelial cells (Rubio-Gayosso *et al.*, 2000). Besides, 17β-estradiol was found to enhance the formation of inositol 1,4,5-triphospate through increased phospholipase C activity (Picotto *et al.*, 1999). In isolated female rat colonic crypts, 17β-estradiol increases $[Ca^{2+}]_i$ by opening of L-type voltage-operated Ca^{2+} channels (Doolan *et al.*, 2000).

1.4.4. Interaction with vasoconstrictors

In addition to the direct effects of estrogens on the vasculature, they may also regulate muscle contractility via interaction with other vasoconstrictors. One of the examples is the interaction of estrogen with renin-angiotensin system, which is targeted therapeutically in the treatment of hypertension. It was reported that estrogen inhibited angiotensin II-induced constrictor effect both *in vitro* and *in vivo*. In isolated tissue studies, chronic estrogen treatment has been shown to down regulate angiotensin

II-receptor expression in the anterior pituitary and adrenal cortex (Carriere *et al.*, 1986). Moreover, angiotensin II-induced contraction was significantly depressed in vascular rings from 17β-estradiol-treated rats, which was independent of the presence of the vascular endothelium (Cheng & Gruetter, 1992). *In vivo* studies showed that 17β-estradiol treatment attenuated contractile responses to exogenous angiotensin II (Magness *et al.*, 1993).

The interaction between estrogen and endothelin-1 was also investigated. Estrogen attenuated endothelin-1-induced vasoconstriction in the isolated rabbit coronary arteries (Jiang *et al.*, 1992a). Moreover, estrogen inhibited endothelin-1 production and secretion from cultured bovine aortic endothelial cell (Morey *et al.*, 1998) and human vascular endothelial cells (Wingrove & Stevenson, 1997). *In vivo* studies, acute administration of physiological concentrations of estradiol attenuated endothelin-1-induced vasoconstriction in both porcine coronary conductance and resistance arteries (Sudhir *et al.*, 1997).

The effects of estrogen on other vasoconstrictors were also examined. Massicotte *et al.* reported that pregnancy reduced pressor responses to arginine 8-vasopressin (Massicotte *et al.*, 1987), it is due to prolong exposure to high level of plasma estrogen. However, there are contradictory results on the norepinephrine response. Although Shan *et al.* found that 17β-estradiol decreased the pressor responses to norepinephrine in male rats (Shan *et al.*, 1994), but enhanced responsiveness to norepinephrine has been described in aortic rings from prepubertal and ovariectomized rats pretreated with 17β-estradiol (Cheng & Gruetter, 1992).

1.4.5. Interaction with endothelium-dependent dilators

As described in Section 1.4.1., endothelium plays a role in estrogen-induced relaxation, the interaction between estrogen and endothelium-dependent dilators will

be discussed. NO, one of the endothelium-dependent dilators, the underlying is the key transducer of a vasodilator message from the endothelium to the underlying vascular smooth muscle cells (Schulz & Triggle, 1994). In cultured bovine endothelial cells (BAEC), acute 17β-estradiol increased the eNOS activity via a Ca^{2+}-dependent mechanism (Goetz *et al.*, 1999). It is proposed that 17β-estradiol induces subcellular translocation of eNOS and this suggests a role of estrogen in the modulation of NO-dependent vascular tone.

It was also reported that estrogen may interact with acetylcholine, another endothelium-dependent vasodilator. Ovariectomized monkeys receiving 17β-estradiol demonstrated a vasodilator response to acetylcholine, while monkeys not receiving hormone replacement showed a vasoconstrictor response to acetylcholine (Williams *et al.*, 1990). In addition, chronic treatment of estrogen increased the dilating sensitivity of the guinea pig coronary microcirculation to acetylcholine (Thompson *et al.*, 2000).

Furthermore, incubation with 17β-estradiol enhanced relaxations to bradykinin, an endothelium-derived vasodilator locally produced by endothelial cells, in isolated human coronary arteries (Barton *et al.*, 1998). However, conflicting results were presented. It is reported that short-term exposure of 17β-estradiol did not affect the endothelium-dependent relaxation induced by bradykinin and A23187 (a calcium ionophore) in porcine coronary artery, both agents stimulate NO synthesis (Teoh *et al.*, 1999).

1.4.6. Interaction with adrenergic response

Estrogen modulates the adrenergically mediated vascular responses. For example, the depressant effect of estrogen treatment on the relaxant response to noradrenaline in the rabbit femoral artery was prevented by the α_2-adrenergic antagonist, rauwolscine (Gisclard *et al.*, 1987). Noradrenaline-induced relaxation of coronary arteries was

enhanced by acute, direct exposure to physiological levels (10^{-9} M) of 17β-estradiol. This enhancement is not due to the effect of 17β-estradiol to suppress α-adrenergic contraction to noradrenaline or to increase coronary β-adrenergic receptor sensitivity (Bell *et al.*, 1995). On the other hand, estrogen replacement in the ovariectomized rats enhanced vasoconstriction induced by endothelial α_2-adrenoceptor activation via an increased release of constricting prostaglandins in mesenteric arteries (Ferrer & Osol, 1998).

The relaxation induced by isoproterenol was impaired in ovariectomized rats and estrogen substitution restored the β-adrenergically-mediated relaxation (Ferrer *et al.*, 1996). In non-vascular smooth muscle from female rabbit detrusor, estrogen treatment resulted in increased relaxant responses mediated by β_2- and β_3-adrenergic receptor subtypes, which may be related to the increased cyclic AMP content (Yono *et al.*, 2000). Estrogen was found to sensitize the isoproterenol-induced rat aortic relaxation probably through nitric oxide- and cytochrome P-450-dependent metabolites (Honda *et al.*, 1998). β-Adrenoceptor-mediated vasorelaxation is thought to be mediated at least in part via cyclic AMP-dependent mechanisms (Gray & Marshall, 1992; Delpy *et al.*, 1996; Toyoshima *et al.*, 1998) or by endothelium-dependent pathway (Graves & Poston, 1993; Huang & Kwok, 1997; Huang *et al.*, 1998). Estrogen-induced vasorelaxation may involve cyclic AMP-mediated pathway (Teoh & Man, 2000). In the cardiac myocytes, a low concentration of 17β-estradiol (1 nM) reduced isoproterenol-induced increase in whole-cell L-type voltage-dependent Ca^{2+} currents (Meyer *et al.*, 1998). It remains, however, elusive whether estrogen potentiation of the isoproterenol-induced relaxation is due to synergistic interaction on cyclic AMP-dependent mechanism or on up-regulation of β-adrenoceptors in vascular smooth muscle cells. If there exists a crosstalk linkage between different intracellular signaling pathways activated by estrogen and β-adrenoceptor agonists, it is reasonable to hypothesize that isoproterenol

may be also able to amplify the relaxant response to 17β-estradiol. Indeed, isoproterenol-induced enhanced responses (heart rate and systolic pressure) to estrogen in rat hearts involved a cyclic AMP-dependent mechanism (Li *et al.*, 2000). These data indicate that estrogen and isoproterenol could mutually exert synergistic interaction with each other. However, the mechanism responsible for β-adrenoceptor-mediated amplification of estrogen-induced vasorelaxation has yet to be explored.

1.5. Clinical Studies

Since the difference in the vascular responses to estrogen may occur between human and animals, various experiments were conducted on the volunteers to clarify whether the effect of estrogen on the human is similar to that in the experimental animals. In experiments investigating the acute effect of estrogen, it was found that intra-arterial infusion of physiological levels of 17β-estradiol selectively potentiated the endothelium-dependent vasodilator response to acetylcholine in the healthy postmenopausal women (Gilligan *et al.*, 1994). Moreover, a significant decrease in cardiac output and heart rate was observed after estrogen administration (Hayward *et al.*, 2000; Luotola *et al.*, 1979). In addition, it was reported that the endothelium-dependent responses to intracoronary 17β-estradiol improved in post-menopausal women, but not in men with coronary atherosclerosis (Collins *et al.*, 1995), indicating that acute estrogen administration appears to benefit the cardiovascular system of postmenopausal women only.

Other experiments were carried out to examine the chronic effect of estrogen. Estrogen replacement therapy increased plasma NO levels and decreased plasma endothelin-1 levels (Wilcox *et al.*, 1997). Moreover, the flow-mediated dilation of the brachial artery increased with the rise in plasma estrogen during the mid-cycle

follicular stage during the ovarian cycle (Hashimoto *et al.*, 1995), indicating that endogenous estrogen affects the vasomotor activity. In addition, an increased level of bradykinin and a concomitant decrease of plasma angiotensin converting enzyme activity were observed after hormone replacement therapy (Sumino *et al.*, 1999). Furthermore, it is found that postmenopausal estrogen replacement therapy raised HDL cholesterol and lower total and LDL cholesterol (Godsland, 2001). These findings are comparable with the results obtained in animal studies, suggesting that the vascular effect of estrogen is quite similar between human and experimented animals.

1.6. Therapeutic Values of Estrogen and Progesterone

Gender difference in the incidence of cardiovascular disease is well documented in literature. In the premenopausal reproductive years, women experience a lower risk of coronary heart disease (CHD) compared with men with the same age. After menopause, this difference significantly narrows unless estrogen replacement therapy is initiated. The epidemiological study demonstrates that the postmenopausal women receiving estrogen therapy have a much lower mortality rate in association with cardiovascular disease. Numerous studies suggested that current estrogen users have a risk of CHD about 50 % lower than that of nonusers, indicating the cardioprotective role of female sex steroid hormones. (Stampfer *et al.*, 1991; Barrett-Connor & Bush, 1991).

Besides, estrogen is used to prevent the occurrence of osteoporosis. It was reported that long-term administration of mestranol (estrogen analogue) prevented bone loss and height loss when comparing with the placebo group (Lindsay *et al.*, 1976; Lindsay *et al.*, 1980).

Moreover, it was found that the age-specific incidence rate of Alzheimer's disease

is nearly twice as high in women as in men (Henderson & Buckwalter, 1994). It is therefore proposed that the estrogen deficiency associated with menopause may contribute to the higher incidence of Alzhemier's disease in women.

Although estrogen replacement therapy reduces the risk of coronary heart diseases, some side effects were reported. Endometrial cancer incidence increased rapidly with use of estrogen treatment (Zeil & Finkle, 1975), however, breast cancer incidence increased only after long-duration use of estrogen (Colditz *et al.*, 1995). Therefore, efforts have been made to reduce this disadvantage by adding progesterone in the therapy, so called hormone replacement therapy. Some literatures described that the incidence of endometrial cancer was reduced by addition of progesterone (The Writing Group for the PEPI Trial, 1995).

On the other hand, estrogen and progesterone are used for birth control. Oral contraceptives contain synthetic estrogen and progestin, which suppress ovulation primarily through inhibition of the hypothalamic-pituitary-ovarian feedback system. In addition, these hormones throughout the cycle create suboptimal endometrial development, as a result, the endometrium would not be suitable for implantation (Pharmaceutical Society of Australia, 1998).

In summary, female steroid hormones have many actions on endothelial cells, vascular smooth muscle cells, osteoblasts, neurons and many other cells. Although administration with such hormones causes some adverse effects, they are still commonly used in hormone replacement therapy for postmenopausal women who experience increasing incidence of cardiovascular disorder.

1.7. Objectives of the Present Study

Cardiovascular disease is rare in premenopausal women compared with men in similar age groups. However, the gender difference in cardiovascular disorder diminishes, and there is an increased incidence of cardiovascular risk in postmenopausal women. Although a number of factors contribute to the development of atherosclerosis, hypertension and coronary heard disease in women, estrogen replacement therapy reduces cardiovascular risk. Some cellular mechanisms have been investigated both *in vivo* and *in vitro*. Cardiovascular benefiting effects of estrogen include anti-hypertensive, hypocholesterolemic, anti-proliferaive and inhibition of oxidation of LDL. Figure 5 summarizes the published cellular and ionic mechanisms by which estrogen exerts its vasodilator effect. Estrogen can target both endothelium and the adjacent vascular smooth muscle cells to reduce the force of contraction. However, many questions remain to be answered in cardiovascular pharmacology of female sex steroid hormones. Therefore, I proposed the following four major objectives in my thesis work:

1. **To investigate the differential role of endothelium and endothelium-derived relaxing factors in the relaxant response to 17β-estradiol and progesterone in isolated rat aortas and mesenteric arteries.**

2. **To investigate the synergistic interaction between 17β-estradiol and β-adrenoceptor agonists of low concentrations and to examine the possible cellular mechanisms responsible for this synergistic action in isolated rat mesenteric arteries.**
3. **To investigate the possible enhancing effect of physiological concentration of 17β-estradiol following acute exposure on β-adrenoceptor agonist-induced vasorelaxation in isolated porcine coronary arteries.**
4. **To investigate the influence of chronic 17β-estradiol deprivation on vascular reactivity in the isolated common carotid arteries in ovariectomized rats.**

All experiments were designed to examine the unexplored effect of female sex steroid hormones in blood vessels. This study should provide some new findings and information regarding pharmacology of 17β-estradiol and progesterone. A long-term impact of this work remains to be confirmed.

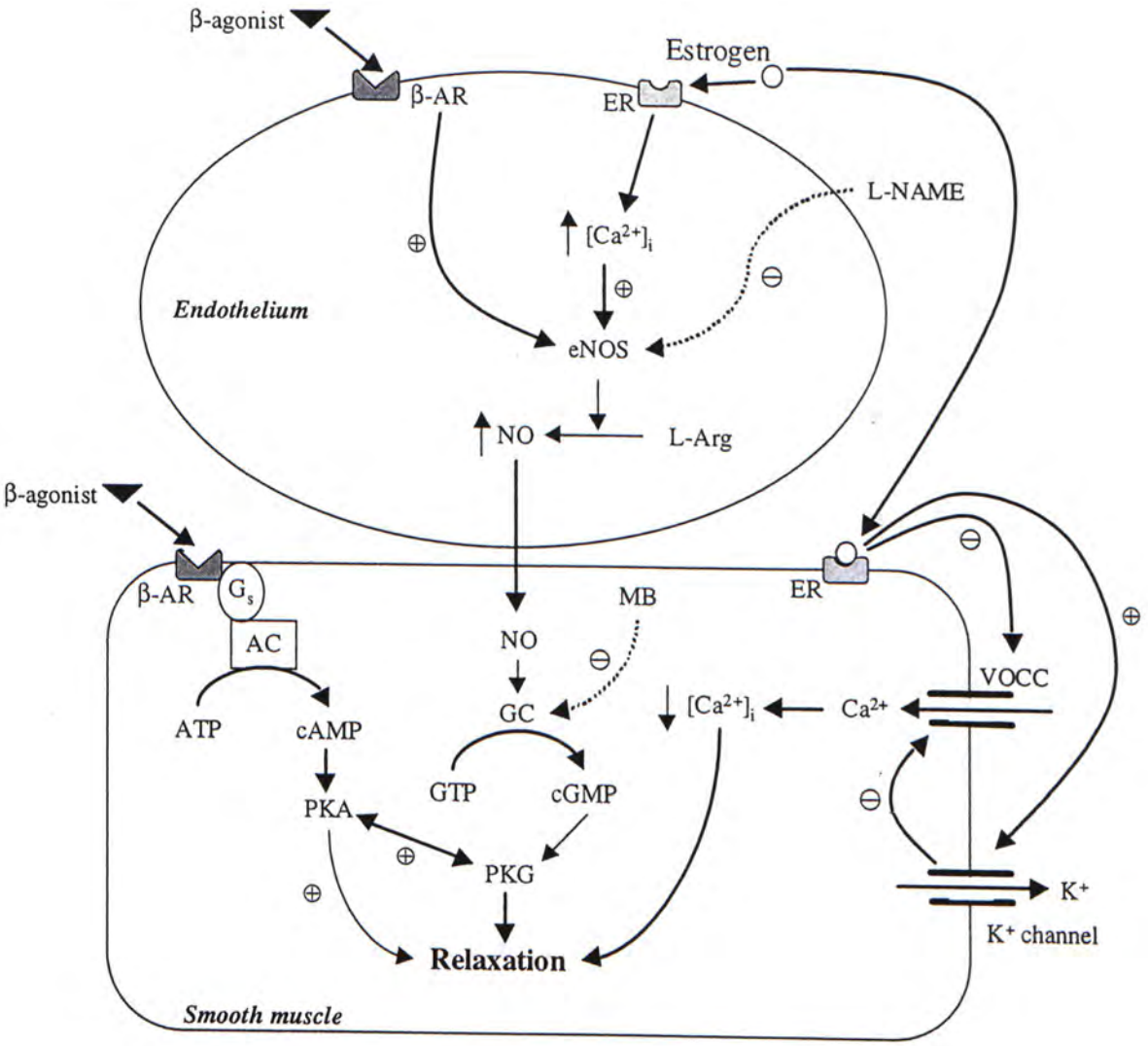

Figure 5

The proposed mechanisms involved in the 17β-estradiol-induced relaxant response in blood vessels. β-adrenoceptor agonists, such as isoproterenol and fenoterol may enhance the 17β-estradiol relaxation. AC, adenylate cyclase; ATP, adenosine triphosphate; β-AR, β-adrenocepter; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; GC, guanylate cyclase; GTP, guanosine triphosphate; L-Arg, L-arginine; L-NAME, N^G-Nitro-L-Arginine methyl ester; MB, methylene blue; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; VOCC, voltage-operated Ca^{2+} channels.

Chapter 2 Methods and Materials

2.1. Tissue Preparation

2.1.1. Preparation of the rat aorta, mesenteric artery and carotid artery

Male or female Sprague-Dawley rats of body weight about 300-350 g (supplied by Animal Services Center, The Chinese University of Hong Kong, Hong Kong) were used in this study. They were killed by pure carbon dioxide. The rat thoracic aorta, mesenteric artery or carotid artery were dissected out and subsequently placed into freshly prepared and oxygenated Krebs-Henseleit solution. Under a light microscope, the surrounding adipose and connective tissues were carefully removed. Each vessel was then cut into ring segments of approximately 4-mm in length for the aorta and 3-mm in length for the mesenteric artery or carotid artery. Three to four rings of thoracic aorta and mesenteric artery, either with endothelium or without endothelium, were prepared from each rat for recording of isometric tension. Each experiment was performed on arterial rings prepared from different rats. All experiments described in this thesis were approved by the Animal Research Ethics Committee, The Chinese University of Hong Kong.

2.1.2. Removal of the functional endothelium

In some experiments, the endothelial layer was mechanically disrupted by gently rubbing the luminal surface of an arterial ring back and forth several times with a plastic tube or small stainless steel wire. Successful removal of the functional endothelium was confirmed by the absence of a relaxant response to acetylcholine (0.1 μM for the aorta, 0.3 μM for the mesenteric artery or carotid artery), an endothelium-dependent vasodilator (Furchgott and Zawadzki, 1980). Endothelium integrity or denudation was also evaluated by light microscopy of the histological section of an artery.

2.2. Organ Bath Set-up

After vessel preparation, each arterial ring was mounted between two stainless steel wires (100 μm in diameter) submerged in an organ bath. One of the steel wires was fixed to a built-in support at the bottom of the organ bath, while other was connected to a force-displacement transducer (FT-03, Grass Instruments Co., Quincy, MA, USA). Changes in isometric force were continuously recorded, and either displayed on a chart recorder or stored by a Maclab software (version 3.5). The diagram in Figure 6 illustrates a set-up for measurement of force of contraction.

Each ring was kept in a 10-ml organ bath containing freshly prepared Krebs-Henseleit solution. The temperature of the bathing solution was maintained at

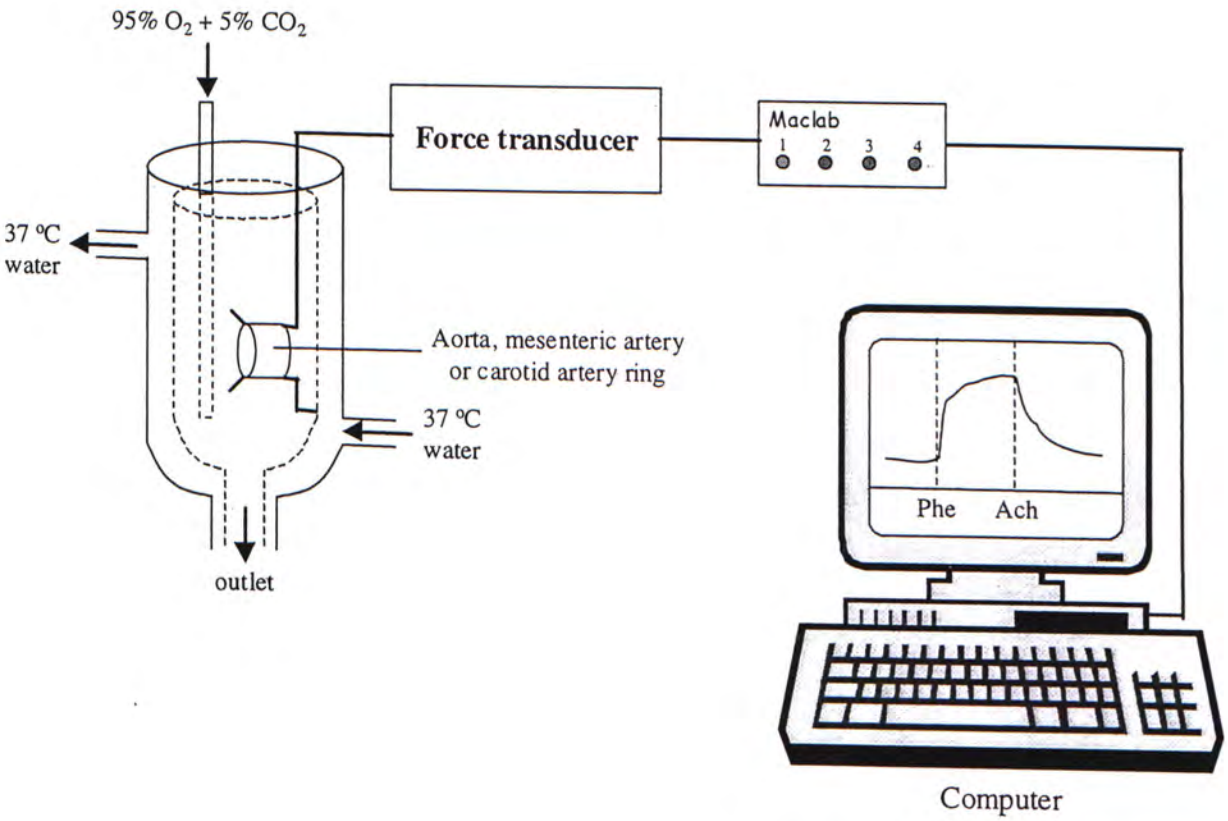

Figure 6

Diagrammatic illustration of isometric force measurement. An artery ring was mounted in an organ bath and changes in contractile force were detected by a Grass force transducer.

37 °C by a heating pump (Polyscience, USA) connected to a circular heat exchanger. The temperature was continuously monitored with a miniature thermometer probe (Edale Instruments Ltd., Cambridge, England). The bath solution was gassed continuously with a mixture of 95% O_2 and 5% CO_2 to give a pH of 7.3-7.4. All solution changes were done through the outlet of the organ bath to a waste container and replacement with a solution pre-warmed to the same temperature.

Arterial rings were suspended optimally under an initial passive resting tension of 1 g for aorta and 0.5 g for mesenteric arteries and carotid arteries. Rings were allowed to equilibrate for about 60 minutes. Within this period of time, the bath solution was replaced with oxygenated and pre-warmed Krebs solution every 20 minutes. The resting tension was readjusted to the optimal resting level, if necessary following each replacement of bath solution.

2.3. Force Measurement

After 60-minute equilibrium, each ring was first contracted with a single concentration of phenylephrine (0.3 μM for aorta and 3 μM for mesenteric arteries or carotid arteries in the presence of endothelium) to test the contractility. Removal of the endothelium was verified by lack of the relaxant response to acetylcholine (0.3 μM for aorta and 1 μM for mesenteric arteries or carotid arteries). Only those

endothelium-intact rings that were relaxed over 85% by acetylcholine were chosen for the present study. Thereafter, the rings were washed several times with Krebs-Henseleit solution until muscle tension returned to the basal tension.

2.3.1. Vascular action of 17β-estradiol and progesterone

2.3.1.1. Role of endothelial nitric oxide in 17β-estradiol- or progesterone-induced relaxation

In this group of experiments, both endothelium-intact and denuded rings were allowed to stabilize for additional 60 minutes under respective resting tension. The rings were contracted by phenylephrine at different concentrations in order to generate muscle tone of similar magnitude in endothelium-intact and –denuded rings (in mesenteric artery: 3 μM with endothelium and 1 μM without endothelium; in aorta: 0.3 μM with endothelium and 0.1 μM without endothelium). After sustained tension (~ 80% of the maximal contractile response to phenylephrine) was obtained, 17β-estradiol or progesterone was added cumulatively to the bath solution. In experiments testing the role of endothelial nitric oxide, rings were exposed for 30 minutes to each of nitric oxide activity inhibitors (N^G-nitro-L-arginine methyl ester and methylene blue) prior to application of phenylephrine. In some experiments, the endothelium-intact rings were first treated with 1 mM L-arginine for 10 minutes, then with 30 μM N^G-nitro-L-arginine

methyl ester before addition of phenylephrine. Since nitric oxide inhibitors enhanced phenylephrine-induced tone, the concentration of phenylephrine was lowered to 0.1 μM for the aortas and 1 μM for the mesenteric arteries.

2.3.1.2. Role of inducible nitric oxide in progesterone-induced relaxation

Apart from the role of endothelial nitric oxide, I also examined the role of inducible nitric oxide in progesterone-induced relaxation. Either endothelium-intact or –denuded mesenteric arteries were exposed for 30 minutes to AMT HCl (5-30 nM). The rings were contracted by 9,11-dieoxy-11_{α},9_{α}-epoxymethanoprostaglandin $F2_{\alpha}$ (U46619) at 30 nM with endothelium or at 10 nM without endothelium in order to generate similar vessel tone. After a sustain contraction, progesterone (0.03-300 μM) was applied cumulatively to induce a concentration-dependent response.

2.3.1.3. Effect of estrogen receptor inhibitor on 17β-estradiol-induced relaxation

After testing the presence of endothelium, the endothelium-intact mesenteric artery rings were exposed for 30 minutes to ICI 182,780 (1-10 μM) before addition of phenylephrine (3 μM). Once sustain contraction was obtained, 17β-estradiol (0.3-300 μM) was applied cumulatively to the bath solution.

2.3.1.4. Interaction between progesterone and 17β-estradiol

In this set of experiments, the endothelium-intact mesenteric arteries were incubated with progesterone (0.3-1 μM) for 2.5 hours. After this period of time, the rings were contracted by U46619 (30 nM). After the contractile response reached a steady level, 17β-estradiol (0.03-300 μM) was added cumulatively to obtain concentration-response curves.

2.3.1.5. Effect of 17β-estradiol on protein kinase C-mediated contraction

In order to gain information on whether 17β-estradiol may interfere with Ca^{2+}-independent contractile mechanisms such as activation of protein kinase C in vascular smooth muscle cells, the effect of 17β-estradiol was examined on phorbol 12,13-diacetate (PDA)-induced sustained increase of muscle tension.

In this set of experiments, the endothelium-denuded mesenteric artery rings were first incubated for 20 minutes in Ca^{2+}-free solution containing 0.3 mM Na_2-EGTA and washed with the same solution twice before addition of 1 μM PDA, the protein kinase activator. Once a sustained tension was obtained, 17β-estradiol or progesterone was added cumulatively (0.1-300 μM) to the bath. In some experiments, the similar procedure was repeated in normal Ca^{2+}-containing Krebs solution.

2.3.1.6. Synergistic interaction between β-adrenoceptor agonists and 17β-estradiol

Relaxations induced by 17β-estradiol were studied in rat isolated mesenteric artery rings contracted by U46619, the concentration of which (10-30 nM) was titrated for each vessel to give a contraction of similar magnitude. When stable tone was obtained, 17β-estradiol was applied cumulatively (0.1 - 100 μM) to the bath solution to determine concentration-response relationships. To examine the potential modulation by β-adrenoceptor activation of 17β-estradiol-induced relaxation, the concentration-dependent relaxing effects for β-adrenoceptor agonists were determined. The concentration that caused approximately 0-10% the maximal relaxation was used. In the first series of experiments, the vessels were exposed to isoproterenol for 1 hr before addition of U46619. In some experiments, the rings were incubated with the β_1- or β_2-adrenoceptor antagonist for 10 minutes before addition of β-adrenoceptor agonist. Some experiments was designed to examine the possible involvement of endothelium-derived relaxing factor (nitric oxide), the effect of isoproterenol was investigated in endothelium-intact rings pretreated with N^G-nitro-L-arginine methyl ester (an inhibitor of nitric oxide synthase) or in endothelium-denuded rings. In the last set of experiments, the endothelium-intact rings were exposed to Rp-cAMPS triethylamine (an inhibitor of cyclic AMP-dependent protein kinase) or Rp8-pCRT-cGMPS triethylamine (an inhibitor of cyclic GMP-dependent protein kinase Iα) for 10 minutes before addition

of isoproterenol. All experiments were carried out in the presence of 10 μM indomethacin to exclude contribution of prostanoids.

2.4. Porcine Coronary Artery Experiments

2.4.1. Vessel preparation

Coronary arteries were obtained from porcine hearts that were harvested in a local slaughterhouse in Hong Kong. Immediately after the pig was killed, the heart was rapidly removed, placed in a container filled with oxygenated Krebs-Henseleit solution at 4 °C, and transferred to the laboratory. Epicardial left circumflex coronary arteries were dissected free of surrounding connective tissues, cut into 3-mm-long rings, and mounted on a pair of stainless steel wires in organ bath (see Section 2.2) filled with Krebs solution at 37 °C.

2.4.2. Force measurement

After 1-hr equilibration, the rings were first contracted with a single concentration of U46619 (3 nM). Removal of the endothelium was verified by the lack of relaxant response to bradykinin (50 nM). In contrast, bradykinin relaxed by over 85% U46619-precontracted arteries, indicating the presence of the functional endothelium.

Thereafter, the rings were washed several times with Krebs-Henseleit solution until muscle tension returned to the basal tension of 2 gram.

2.4.3. Experimental protocol

2.4.3.1. Effect of physiological level of 17β-estradiol on β-adrenoceptor agonist-induced relaxation

The rings of endothelium-intact porcine coronary arteries were first exposure to physiological concentrations of 17β-estradiol (0.1-1 nM) for either 20 minutes or 1 hour. The rings were then contracted with U46619 (3 nM) to establish a sustained contraction. After the contraction had reached a steady level, each of three β-adrenoceptor agonists: isoproterenol, fenoterol or dobutamine was applied to the organ bath cumulatively to induce concentration-dependent responses.

In some experiments, the inhibitory effect of tamoxifen was investigated. 10 μM tamoxifen was added 10 minutes prior to application of 17β-estradiol. Then the protocol was similar to the above to obtain concentration-response curve.

2.4.3.2. Effect of physiological level of 17β-estradiol on phosphodiesterase inhibitor-induced relaxation

The endothelium-intact porcine coronary artery rings were first exposed to 17β-estradiol (0.1-1 nM) for either 20 minutes or 1 hour. They were then contracted

with U46619 (3 nM). After the contractile response reached a steady level, 3-isobutyl-1-methylxathine (IBMX) (0.01-300 μM) was applied to the organ bath cumulatively to induce concentration-dependent responses.

2.5. Ovariectomy

2.5.1. Method of ovariectomy

Menopause was induced surgically in female Sprague-Dawley rats by bilateral ovariectomy. About fifty rats (200-250 g), aged between 3 and 4 weeks, were anesthetized intraperitoneally with 2.5% sodium pentobarbitol (0.16 ml/100 g). The ovaries were ligated and then removed. Following surgical operation, rats were then allowed to recover for 14 days with food and water, and were kept in a 12:12-hr light/dark cycle. At day 15, 17β-estradiol-impregnated timed-released pellets (0.5 mg/pellet; 21-day release, Innovative Research of America) were implanted subcutaneously in the estrogen-replaced group (n=10). The second group of rats (n=10) underwent ovariectomy but received no estrogen replacement. Tamoxifen citrate time-released pellets (0.5 mg/pellet; 21-day release, Innovative Research of America) were implanted subcutaneously in the third group (n=10). In the forth group (n=10), the rats were implanted with both of 17β-estradiol and tamoxifen pellets. These four groups of rats were kept under the same conditions together with a sham-operated

group (or control group) (n=10) (Figure 7). Tail-cuff systolic pressure and heart rate were measured every week and body weight was simultaneously recorded. The rats were killed by cervical dislocation three weeks after the pellet implantation. The serum for β-estradiol level was obtained by collection of trunk blood the blood from abdominal aorta followed by centrifugation. The weights of the uterus were also recorded. The plasma estradiol levels were determined by an ELISA.

2.5.2. Preparation of blood vessels

The rats were killed by cervical dislocation and the common carotid arteries from both sides were dissected out under a sterile condition, and placed in Krebs-Henseleit solution that had been bubbled with a gas mixture of 95% O_2 plus 5% CO_2. Each artery was cleaned off the surrounding connective tissues, and then cut into ring segments of approximately 3-mm in length. In some experiments, the endothelium layer was mechanically disrupted by gently rubbing the luminal surface of an arterial ring back and forth several times with plastic tubing. The removal of endothelium was verified by failure of rings to relax in response to acetylcholine. All rings were equilibrated for 60 minutes before the experiment commenced.

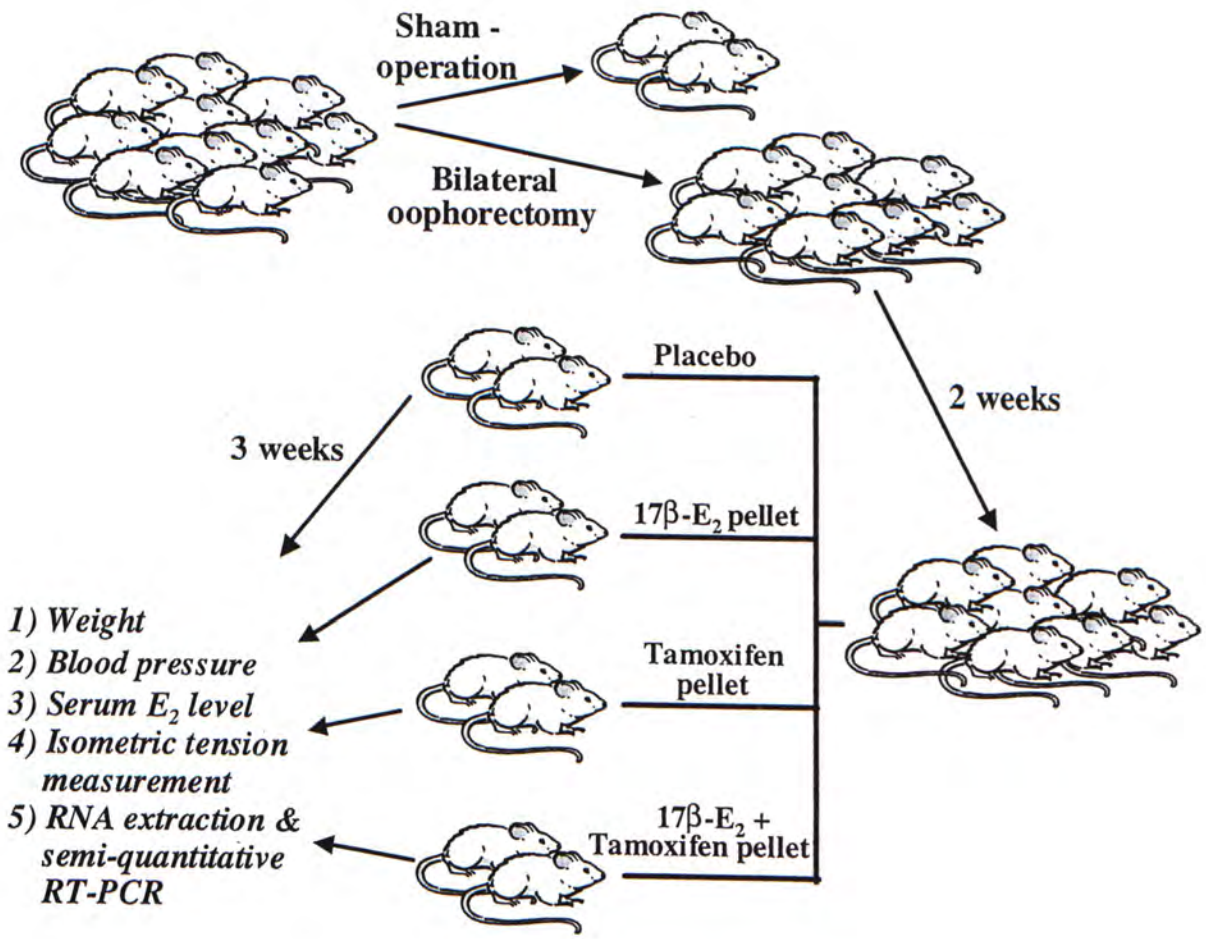

Figure 7

Diagrammatic illustration of ovariectomy experiments. There are five groups: Control, ovariectomized, estrogen-replaced, tamoxifen-replaced, co-treatment of estrogen and tamoxifen group. 17β-E_2, 17β-estradiol.

2.5.3. Experimental protocols

2.5.3.1. Effect of ovariectomy on contractility of rat carotid arteries

Following the equilibration period, both endothelium-intact and -denuded carotid arteries were contracted by adding phenylephrine (0.001-10 μM) and U46619 (1-50 nM) cumulatively. Endothelium-denuded arteries were contracted by high extracellular K^+ (5-80 mM) cumulatively. All the results were expressed in term of actual force (mN).

2.5.3.2. Effect of ovariectomy on relaxation properties of rat carotid arteries

After 60-min equilibration period, the endothelium-intact carotid arteries were contracted by 30 nM U46619. Once the sustained tension was reached, acetylcholine was added cumulatively (0.001-30 μM) to induce concentration-dependent relaxation.

2.6. Chemicals and Solutions

Table 2 contains a list of chemicals and drugs used in the present study. They were purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA), RBI (Research Biochemicals International, Natick, MA, USA), Tocris (Tocris Cookson Ltd., Bristol, UK). The chemical safety for using these compounds was approved by the Safety Office, The Chinese University of Hong Kong, Hong Kong.

Table 2 ***Chemicals and drugs***

Chemicals	Description	Solvent	Source
17β-estradiol pellet	17β-estradiol-impregnated time-released pellet (0.5 mg/pellet; 21-day release)	—	Innovative Research of America
3-Isobutyl-1-methylxathine	Potent phosphodiesterase inhibitor; more active than theophylline at adenosine receptors	DMSO	Sigma
9,11-Dideoxy-11$_\alpha$,9$_\alpha$-epoxymethano-prostaglandin $F_{2\alpha}$	Thromboxane A_2 analogue vasoconstrictor	DMSO	Sigma
(±)-Atenolol	Selective β_1-adrenoceptor antagonist	DMSO	RBI
Acetylcholine	Muscarinic receptor agonist; Endothelium-dependent vasodilator	H_2O	Sigma
AMT HCl	Potent, selective and reversible inhibitor of inducible nitric oxide synthase	H_2O	RBI
Bradykinin	Neuropeptide which contracts smooth muscle, dilates peripheral vessels through endothelium-derived hyperpolarizing factor (EDHF)-induced pathways, increase capillary permeability	H_2O	RBI
β-estradiol	Estrogen, sex hormone	DMSO	RBI
Dobutamine hydrochloride	β_1-adrenoceptor agonist	H_2O	Tocris
Fenoterol hydrobromide	β_2-adrenoceptor agonist	H_2O	Sigma

Forskolin	Adenylate cyclase activator	DMSO	RBI
ICI 118, 551	Selective β_2-adrenoceptor antagonist	H_2O	RBI
ICI 182, 780	Estrogen receptor antagonist	Tocris	RBI
(±)-Isoproterenol HCl	Non-selective β-adrenoceptors agonist	H_2O	RBI
L-Arginine	Nitric oxide precursor	H_2O	Sigma
Methylene blue	Inhibitor of guanylate cyclase	H_2O	Sigma
N^G-nitro-L-arginine methyl ester hydrochloride	Competitive inhibitor of nitric oxide synthase	H_2O	RBI
Phenylephrine HCl	α_1-adrenoceptor agonist, vasoconstrictor	H_2O	RBI
Progesterone	Female sex steroid hormone	DMSO	RBI
DL- Propranolol	Non-selective β-adrenoceptor antagonist	DMSO	Sigma
Phorbol 12,13-diacetate	Protein kinase C activator, vasoconstrictor	DMSO	RBI
Rp-cAMPS triethylamine	Specific inhibitor of cAMP-dependent protein kinase I and II	H_2O	Sigma
Rp-8-pCRT-cGMPs triethylamine	Inhibitor of cGMPs-dependent protein kinase Iα	H_2O	RBI
Sodium pentobarbitol	Anesthetic	H_2O	Sigma
Tamoxifen citrate	Anti-estrogen	DMSO	RBI
Tamoxifen pellet	Tamoxifen citrate time-released pellets (0.5 mg/pellet; 21-day release0	—	Innovative Research of America

The drug stock solution was normally prepared and stored in distilled water or DMSO. Further dilution was made when necessary, in distilled water or DMSO to a desired concentration. β-estradiol, fenoterol hydrobromide, (±)-isoproterenol HCl, methylene blue, phorbol 12, 13-diacetate are light-sensitive, the stock solution was protected from light stimulation.

Table 3 contains compositions of three different solutions used in my study. They were freshly prepared before the experiments.

Table 3 *Compositions of Solutions*

	Krebs-Henseleit (mM)	Ca^{2+} free (mM)	60 mM K^+ (mM)	60 mM K^+ Ca^{2+} free (mM)
NaCl	119	119	63	63
$NaHCO_3$	25	25	25	25
$MgCl_2 \cdot 6H_2O$	1	1	1	1
KCl	4.7	4.7	60	60
KH_2PO_4	1.2	1.2	1.2	1.2
$CaCl_2$	2.5	0	2.5	0
D-glucose	11.1	11.1	11.1	11.1
Na_2-EGTA	—	0.3	—	—

The pH value of normal Krebs, Ca^{2+} free and 60 mM K^+ solutions was maintained at 7.3-7.4 when the bath solution was continuously oxygenated with a mixture of 95% O_2 plus 5% CO_2 at 37 °C.

2.7. Statistical Analysis

Data are presented as means ± standard errors of the mean (S.E.M.) and *n* refers to the number of arterial rings (animals) examined. The relaxant responses to 17β-estradiol or progesterone are expressed as percentage reversal of the vasoconstrictor-induced contraction. In Section 3.1. to 3.4., IC_{50} values were calculated as the drug concentration causing the half maximum relaxation. These values were compared in the absence and presence of various pharmacological agents. Cumulative concentration-relaxation relations were analyzed with non-linear curve fitting by means of a logistic equation (Grafit, Erithacus Software Ltd.). In Section 3.5.-3.7., the negative logarithm (-log) of the concentration of a drug required to produce 50% (pD_2) the maximal relaxation was calculated by non-linear regression curve fitting (Graphpad Prism Software, version 3.0, USA). Statistical analysis was performed by using Student's t-test (two-tailed) or one-way analysis of variance (ANOVA) followed by Newman-Keuls test when more than two groups were compared. A P value < 0.05 was taken to indicate a significant difference.

Chapter 3 Results

3.1. Role of Endothelium/Nitric Oxide in 17β-Estradiol- and Progesterone-induced Relaxations

3.1.1. Relaxant response of 17β-estradiol

As shown in Figure 8, 1 μM 17β-estradiol caused approximately 20% relaxation in an isolated endothelium-intact aorta (Figure 8a), but not in an endothelium-denuded aortic ring (Figure 8b). In the phenylephrine-contracted isolated rat endothelium-intact aortas, 17β-estradiol induced concentration-dependent relaxations with an IC_{50} of 2.28 ± 0.14 μM (n=8). Removal of the functional endothelium significantly reduced the relaxing potency of 17β-estradiol with an IC_{50}: 8.13 ± 0.51 μM (n=6, $P < 0.05$ compared with that obtained with endothelium). Figure 9a shows that the concentration-response curve for 17β-estradiol-induced relaxation was shifted to the right in the endothelium-denuded aortas. However, the results are different in the isolated rat mesenteric arteries. 17β-estradiol also concentration dependently decreased the phenylephrine-induced tone in rat mesenteric artery rings, but this relaxant response was unrelated to the presence of endothelium. The IC_{50} values are 6.37 ± 1.4 μM (n=5) and 5.29 ± 0.79 μM (n=4) for the relaxant effect of 17β-estradiol in rings with and without endothelium, respectively ($P > 0.05$, Figure 9b). A similar magnitude of vessel tone was produced by titrating phenylephrine concentration in both endothelium-intact and –denuded rings.

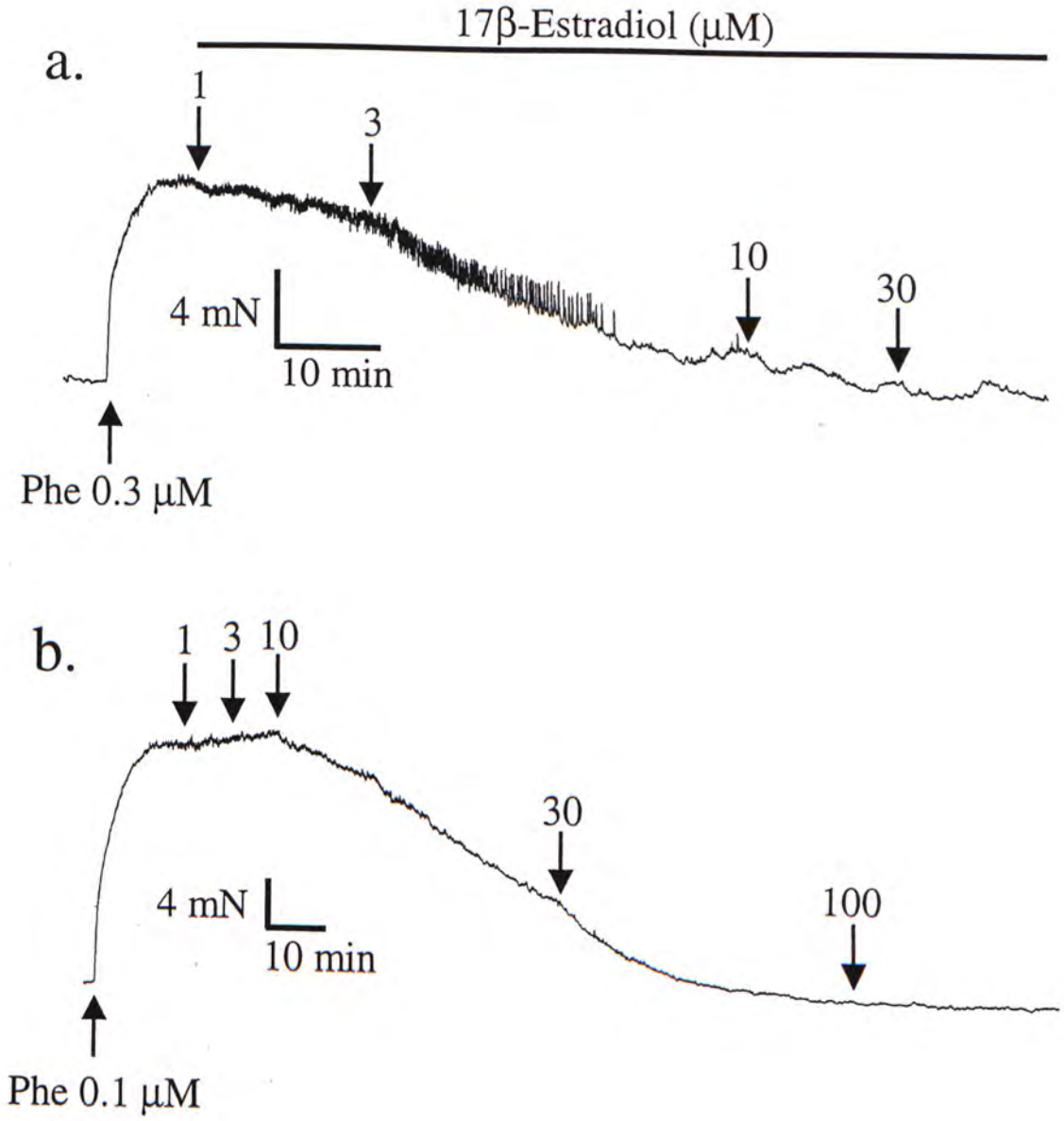

Figure 8

The concentration-dependent relaxations induced by 17β-estradiol in phenylephrine-contracted rat aortic rings with endothelium (**a**) and without endothelium (**b**).

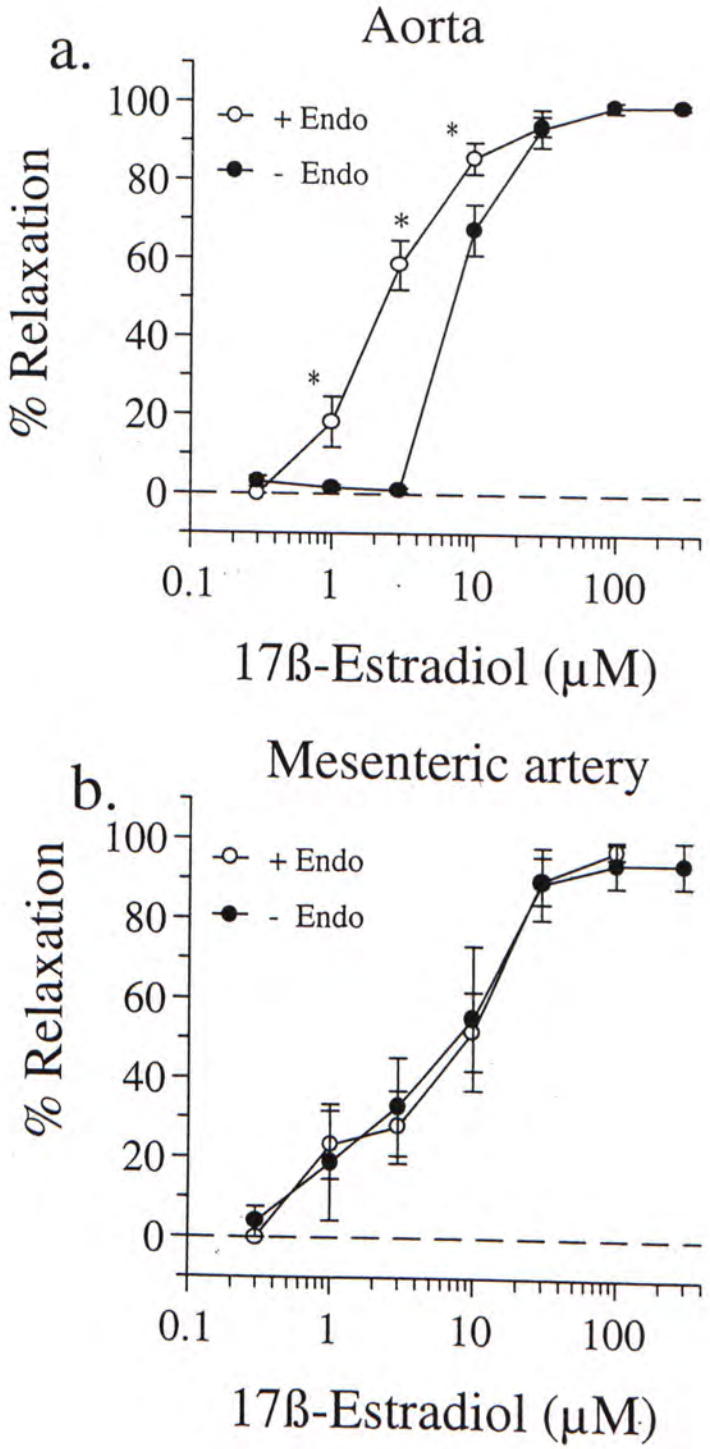

Figure 9

Concentration-response curves for the relaxant response to 17β-estradiol in the rat aortas (**a**: ○, n=8 with endothelium; ●, n=6 without endothelium) or in the mesenteric arteries (**b**: ○, n=5 with endothelium; ●, n=4 without endothelium). The data are means ± S.E.M. of *n* experiment. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

3.1.2. Effects of inhibitors of nitric oxide activity on 17β-estradiol-induced relaxation

The concentration-dependent relaxant effect of 17β-estradiol (Figure 9a) was significantly greater in the endothelium-intact than endothelium-denuded aortas, indicating that the endothelium-derived vasoactive factors are involved.

Pretreatment of endothelium-intact aortic rings with 30 μM N^G-nitro-L-arginine methyl ester (L-NAME) inhibited 17β-estradiol-induced relaxation, while 1 mM L-arginine reversed the effect of L-NAME (Figure 10a). Moreover, pretreatment of methylene blue (10 μM) which is an inhibitor of guanylate cyclase, also significantly attenuated the 17β-estradiol-induced relaxation to the same degree as that seen with L-NAME in Figure 10a (Figure 10b). These data favour a role of endothelium/nitric oxide in the 17β-estradiol-induced relaxation in the rat aortas.

3.1.3. Relaxant response of progesterone

The concentration-dependent relaxant effect of progesterone was significantly greater in an endothelium-intact (Figure 11a) than endothelium-denuded mesenteric artery (Figure 11b), indicating that the endothelium-derived vasoactive factors may be involved. Figure 12b shown that progesterone induced relaxation with greater effectiveness in endothelium-intact mesenteric artery rings than endothelium-denuded rings. The IC_{50} values are 2.63 ± 0.48 μM (n=9) and 8.58 ± 1.17 μM (n=6) for the relaxant effect of 17β-estradiol in mesenteric arteries with and without endothelium, respectively ($P < 0.05$). However, this endothelium-dependent effect was not observed in the rat aortas. Progesterone relaxed the phenylephrine-preconstricted

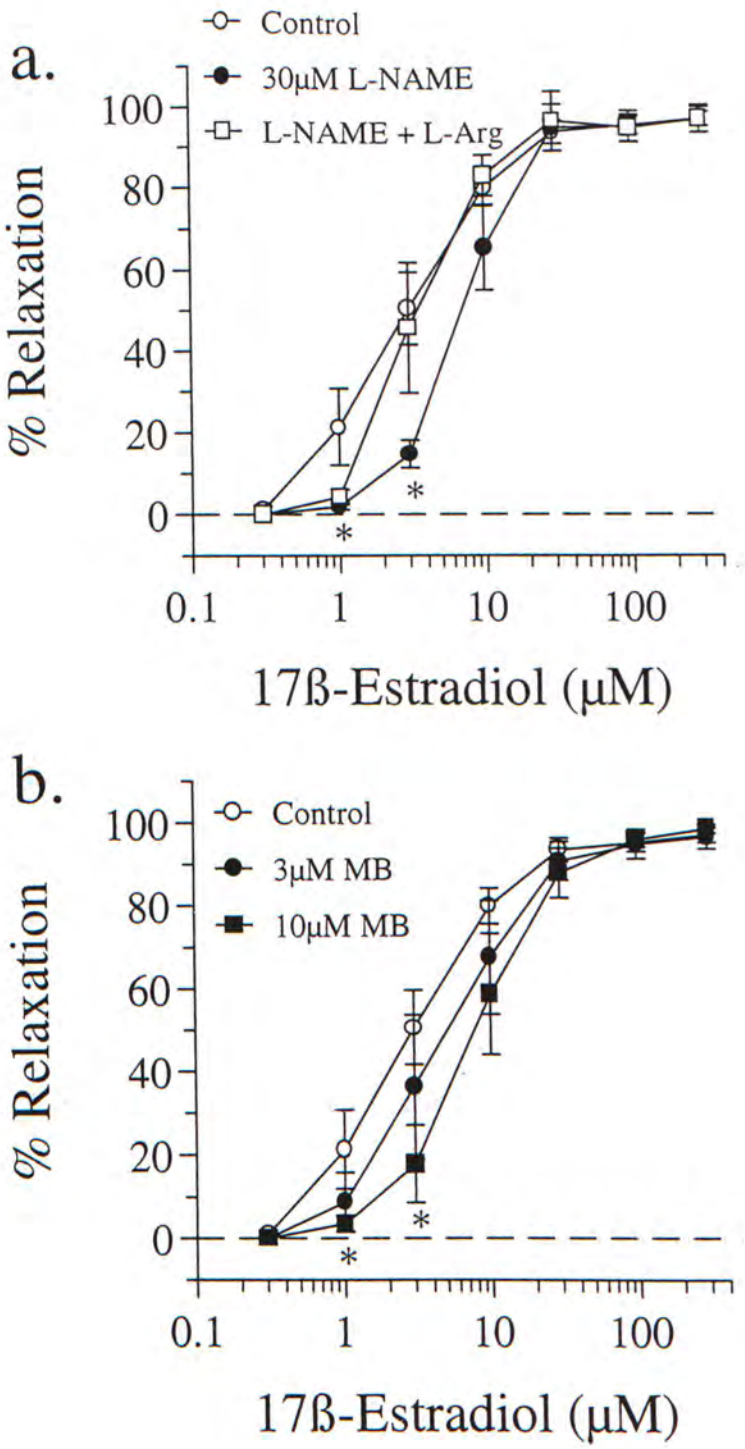

Figure 10

(**a**) Concentration-response curves for 17β-estradiol-induced relaxation in rat endothelium-intact aortas (○, n=9 for control; ●, n=5 for 30 μM L-NAME; □, n=4 for 1 mM L-arginine plus 30 μM L-NAME). (**b**) Concentration-response curves for 17ß-estradiol in rat endothelium-intact aortas (○, n=9 for control; ●, n=5 for 3 μM MB; ■, n=5 for 10 μM MB). The data are means ± S.E.M. of *n* experiments. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

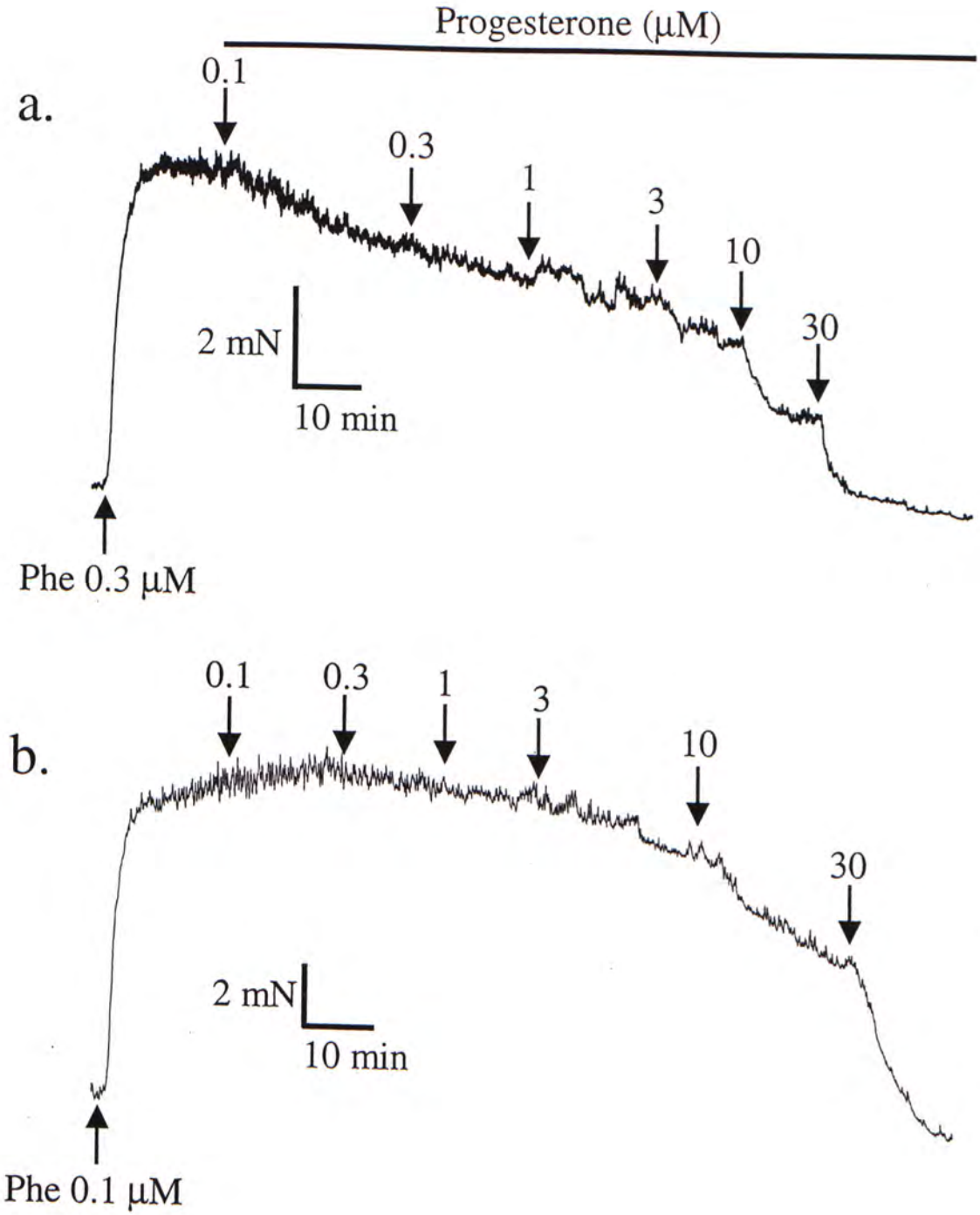

Figure 11

The concentration-dependent relaxations induced by progesterone in phenylephrine-contracted rat aortic rings with endothelium (**a**) and without endothelium (**b**).

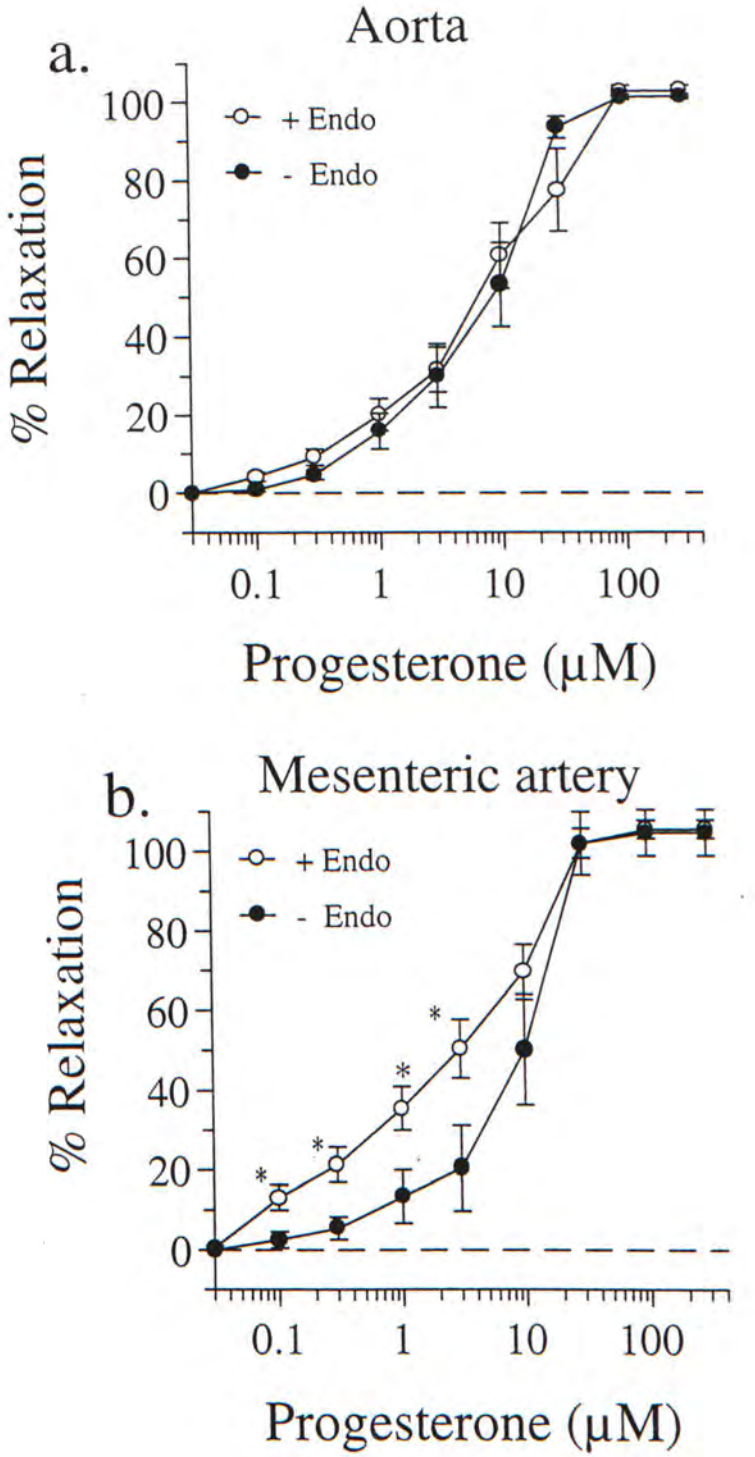

Figure 12

Concentration-response curves for progesterone-induced relaxation in rat aortas (**a**) and mesenteric arteries (**b**) with endothelium (○, n=7-9) and without endothelium (●, n=6-10). The data are means ± S.E.M. of *n* experiments. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

endothelium-intact aortas with an IC_{50} of 6.35 ± 0.77 μM (n=7), and this value was similar to that obtained in endothelium-denuded rings (IC_{50}: 6.68 ± 0.85 μM, n=10, $P > 0.05$) (Figure 12a).

3.1.4. Effects of inhibitors of nitric oxide activity on progesterone-induced relaxation

Progesterone-induced relaxation in endothelium-intact mesenteric artery rings was significantly attenuated by pretreatment with 30 and 100 μM L-NAME (Figure 13a). L-arginine at 1 mM partially reversed the inhibitory effect of 30 μM L-NAME on progesterone-induced relaxation (Figure 13a). In addition, pretreatment with 3 μM methylene blue also caused rightward shift of the concentration-response curve for progesterone-induced relaxation (Figure 13b). The percentage relaxation induced by acetylcholine and initial vessel tension are presented in Table 4 and the IC_{50} values from Figure 8-13 were summarized in Table 5.

In addition, the effect of AMT HCl, an inhibitor of inducible nitric oxide synthase, was also examined. In this series of experiments, pretreatment of rings with AMT HCl at three concentrations (5, 10 and 30 nM) inhibited the progesterone-induced relaxation in rat endothelium-intact mesenteric arteries (Figure 14a), indicating that 30 nM is the concentration at which AMT HCl maximally inhibited the progesterone-induced relaxation. However, exposure of endothelium-denuded rings to the same concentrations (5-30 nM) of AMT HCl did not cause shifts of the concentration-response curve (Figure 14b). AMT HCl at 30 nM slightly enhanced progesterone-induced relaxation (Figure 14b). The IC_{50} values from Figure 14 were summarized in Table 6.

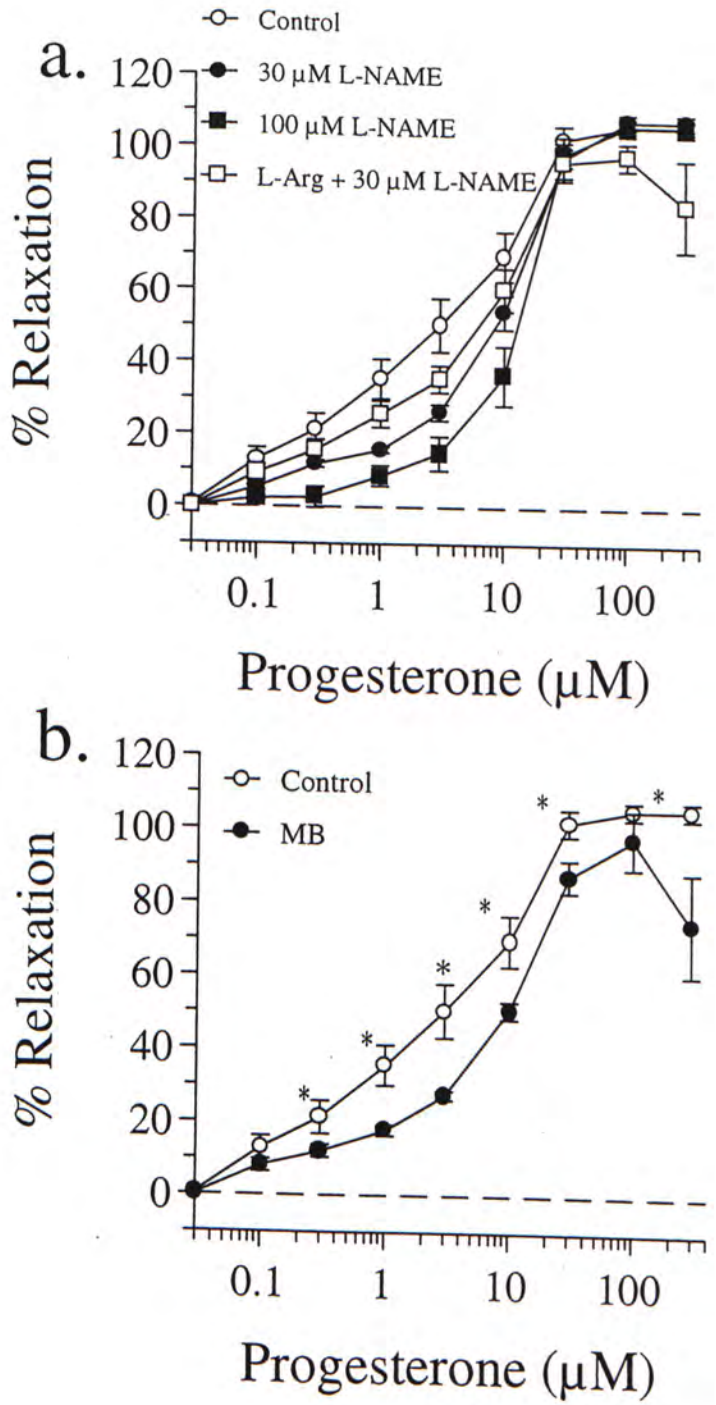

Figure 13

(a) Concentration-response curves for progesterone-induced relaxation in the rat endothelium-intact mesenteric arteries (○, n=9 for control; ●, n=8 for 30 μM L-NAME; □, n=6 for 1 mM L-arginine plus 30 μM L-NAME; ■, n=5 for 100 μM L-NAME). **(b)** Concentration-response curves for progesterone-induced relaxation in rat endothelium-intact mesenteric arteries (○, n=9 for control; ●, n=5 for 3 μM MB). The data are means ± S.E.M. of *n* experiments. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

Table 4 ***Acetylcholine-induced relaxation and phenylephrine-induced initial tone of endothelium-intact rings in different treatment groups***

	17β-Estradiol in aortas			Progesterone in mesenteric arteries		
Groups	Relaxation (%)	Initial tone (mN)	n	Relaxation (%)	Initial tone (mN)	n
Control	78.3 ± 10.0	15.1 ± 0.9	8	92.0 ± 3.0	8.1 ± 1.1	9
L-NAME 30 μM	76.3 ± 4.1	17.7 ± 1.4	5	84.1 ± 9.8	8.2 ± 0.6	8
L-NAME 100 μM				90.7 ± 4.8	7.6 ± 0.9	5
L-Arg + L-NAME	80.4 ± 5.3	16.1 ±1.3	4	94.6 ± 3.2	8.6 ± 0.7	6
MB 3 μM	79.3 ± 3.8	14.3 ± 2.8	5	88.8 ± 1.8	8.5 ± 0.6	5
MB 10 μM	75.1 ± 2.6	16.8 ± 0.9	5			

Acetylcholine-induced relaxation in phenylephrine-preconstricted rings was taken as an index of the presence of the functional endothelium and this value was not different among various treatment groups. Following confirmation of the integrity of endothelium, the initial tone induced by phenylephrine (0.1-3 μM) was similar among different groups. Statistical analysis was performed using one-way ANOVA followed by Student-Newman-Keuls test.

Table 5 *Effects of nitric oxide inhibitors on relaxation induced by 17β-estradiol and progesterone*

	17β-Estradiol in aortas		Progesterone in mesenteric arteries	
	IC_{50} (μM)	n	IC_{50} (μM)	n
+Endothelium				
Control	2.28 ± 0.14	8	2.63 ± 0.48	9
L-NAME 30μM	6.94 ± 0.36[a]	5	7.54 ± 0.71[a]	8
L-NAME 100 μM	—		12.3 ± 1.17[a]	5
L-Arginine 1 mM + L-NAME 30 μM	3.37 ± 0.19[b]	4	4.02 ± 0.90[b]	6
MB 3 μM	4.80 ± 0.27[a]	5	6.64 ± 0.72[a]	5
MB 10 μM	7.83 ± 0.73[a]	5	—	
-Endothelium	8.13 ± 0.51[a]	6	8.58 ± 1.17[a]	6

IC_{50} values for the relaxant response to 17β-estradiol in the isolated rat aortas and to progesterone in the mesenteric artery rings. Statistical difference ($P < 0.05$) was indicated between control and treatment groups ([a]) or between L-NAME group and L-Arg + L-NAME group ([b]). L-Arg, L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; MB, methylene blue. Results are means ± S.E.M. of *n* experiments.

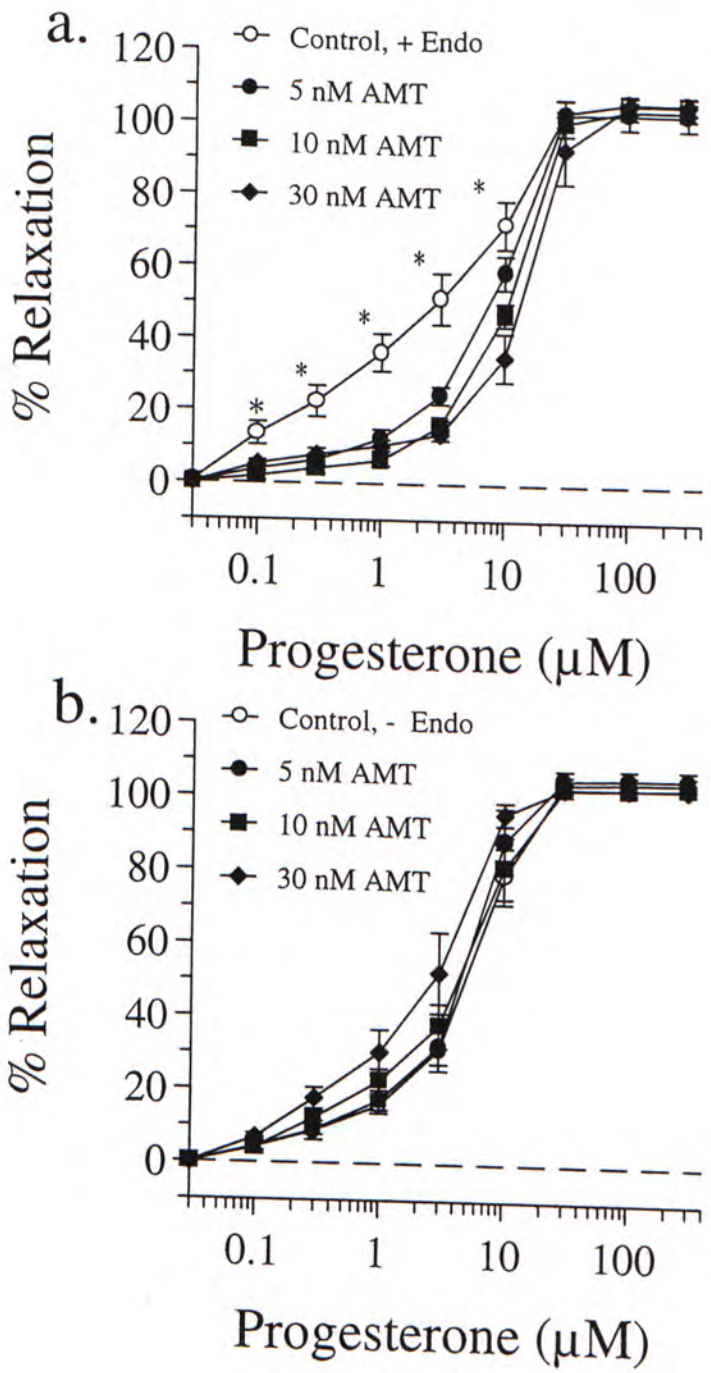

Figure 14

The effects of AMT on progesterone-induced relaxation in rat mesenteric arteries (**a**) with endothelium (○, n=12 in control; ●, n=6 in 5 nM AMT; ■, n=5 in 10 nM AMT and ◆, n=6 in 30 nM AMT) and (**b**) without endothelium (○, n=7 in control; ●, n=6 in 5 nM AMT; ■, n=8 in 10 nM AMT and ◆, n=5 in 30 nM AMT). Results are means ± S.E.M. of *n* experiments. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

Table 6 *Effect of AMT HCl on the relaxation induced by progesterone*

	With endothelium		Without endothelium	
	IC_{50} (μM)	n	IC_{50} (μM)	n
Control	3.62 ± 1.28	12	5.38 ± 0.57	7
5 nM AMT HCl	8.13 ± 1.17*	6	4.81 ± 0.52	6
10 nM AMT HCl	10.89 ± 1.02*	5	4.43 ± 0.68	8
30 nM AMT HCl	14.37 ± 1.27*	6	2.68 ± 0.5	5

IC_{50} values for the relaxant responses to progesterone in the isolated rat mesenteric artery rings with and without the functional endothelium. Statistical difference between control and treatment groups is indicated by asterisk* ($P < 0.05$). Results are means ± S.E.M. of *n* experiments.

3.2. Effect of Estrogen Receptor Inhibitor on 17β-Estradiol-induced Relaxation

The presence of plasma membrane estrogen receptors was reported before (Pietras & Szego, 1977), the effect of the estrogen receptor inhibitor was therefore investigated. In endothelium-intact mesenteric arteries, the concentration-response curve to 17β-estradiol was not affected by pretreatment with the estrogen receptor antagonist, ICI 182,780 (1-10 μM). The IC_{50} values are 4.79 ± 0.07 μM for control; 4.36 ± 0.11 μM for 1 μM; 3.29 ± 0.03 μM for 3 μM; 3.77 ± 0.04 μM for 10 μM ($P > 0.05$, Figure 15).

3.3. Interaction between Progesterone and 17β-Estradiol

The effect of progesterone was investigated in both rat endothelium-intact and endothelium-denuded mesenteric arteries. It was found that concentration-response curve for 17β-estradiol was shifted to the left when the endothelium-intact rings were pretreated for 2.5 hours with 0.3 or 1 μM progesterone (Figure 16a). The IC_{50} values are 10.14 ± 0.05 μM for control; 3.28 ± 0.09 μM for 0.3 μM progesterone; 2.82 ± 0.11 μM for 1 μM progesterone ($P < 0.05$, compared with the control value). However, progesterone did not affect 17β-estradiol-induced relaxation in endothelium-denuded tissues (Figure 16b). The IC_{50} values are 7.24 ± 0.05 μM for control and 5.82 ± 0.04 μM for 0.3 μM progesterone ($P > 0.05$, compared with the control value).

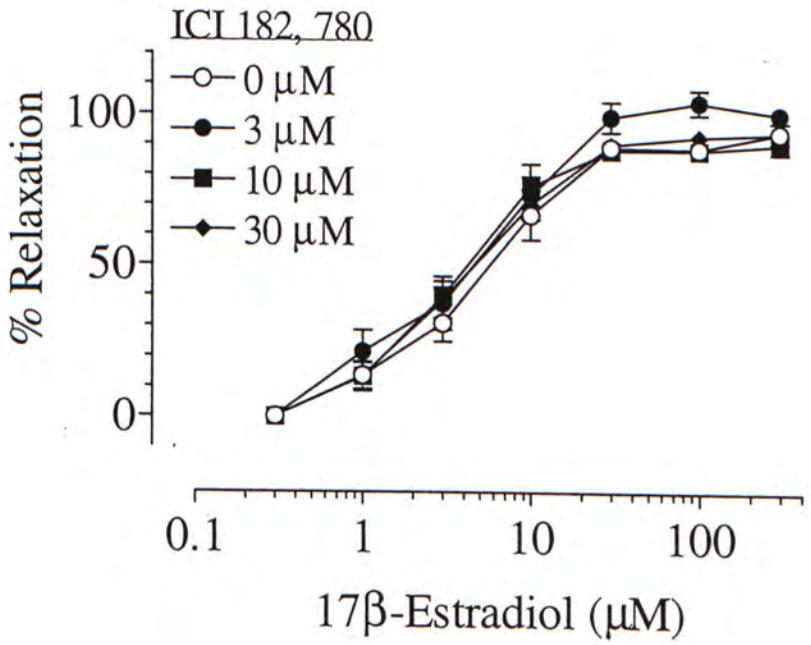

Figure 15

The effect of ICI 182,780 on progesterone-induced relaxation in rat mesenteric arteries with endothelium (○, n=4 in control; ●, n=5 in 1 μM; ■, n=3 in 3 μM and ◆, n=5 in 10 μM). Results are means ± S.E.M. of *n* experiments.

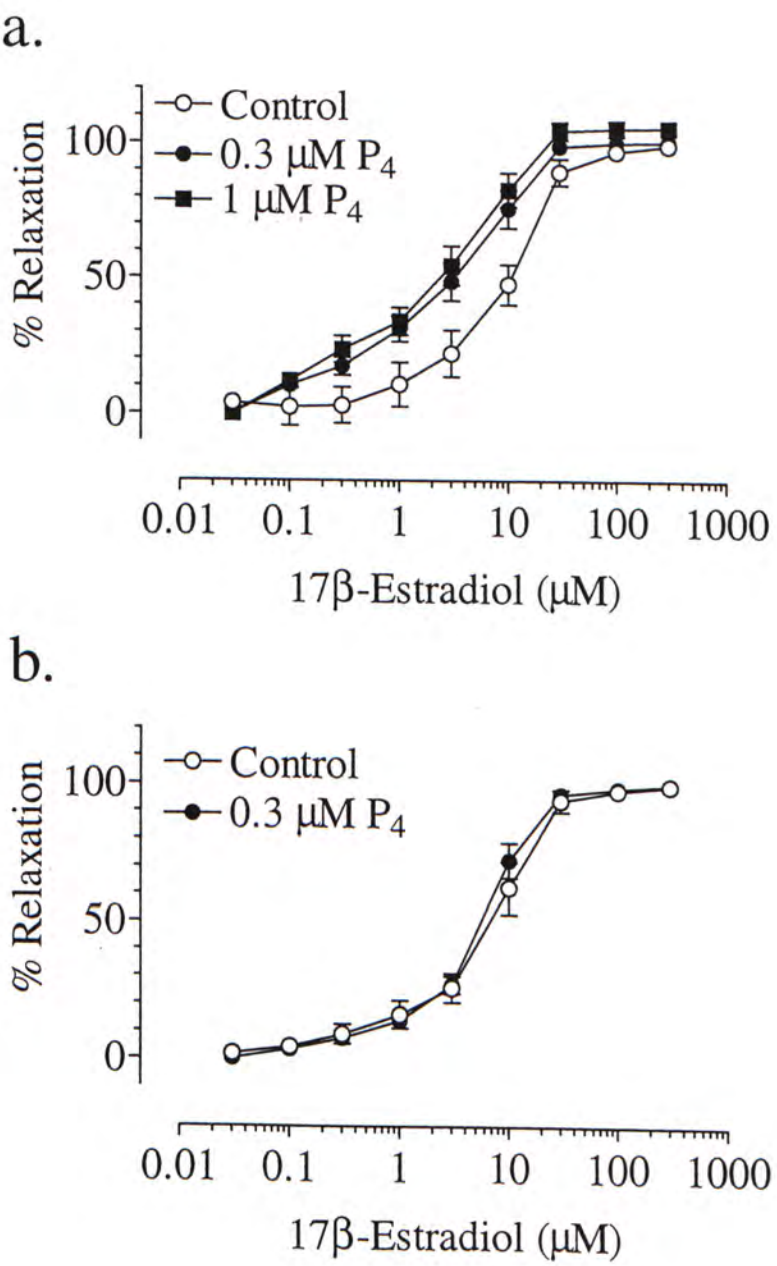

Figure 16

The effect of progesterone (P_4) on the 17β-estradiol relaxation in rat mesenteric artery rings. **(a)** Concentration-response curves for 17β-estradiol in endothelium-intact tissue (○, n=7 for control; ●, n=6 for 0.3 μM progesterone; ■, n=6 for 1 μM progesterone). **(b)** Concentration-response curves for 17β-estradiol in endothelium-denuded rings (○, n=6 for control; ●, n=8 for 0.3 μM progesterone). Results are means ± S.E.M. of *n* experiments.

3.4. Effect of Female Sex Steroid Hormones on Protein Kinase C-mediated Contraction

3.4.1. Effect of 17β-estradiol on phorbol ester-induced contraction

In the phorbol 12, 13-diacetate (PDA, 1 μM)-contracted isolated rat endothelium-denuded mesenteric arteries, 17β-estradiol (0.1-300 μM) induced concentration-dependent relaxations with an IC_{50} of 11.67 ± 5.43 μM (n=6) in normal Krebs solution. In the Ca^{2+}-free Krebs solution, the relaxing potency of 17β-estradiol was enhanced with an IC_{50} of 3.41 ± 0.4 μM (n=6, $P < 0.05$). Figure 17 shows that the concentration-response curve for 17β-estradiol was shifted to the left when the experiments were repeated in Ca^{2+}-free Krebs solution. The vessel tension induced by 1 μM PDA was 11.3 ± 0.5 mN and 10.7 ± 0.6 mN in normal and Ca^{2+}-free Krebs solution, respectively.

3.4.2. Effect of progesterone on phorbol ester-induced contraction

In the isolated rat endothelium-denuded mesenteric arteries, progesterone (0.1-300 μM) induced concentration-dependent relaxations in the PDA (1 μM)-precontracted rings in either normal Krebs solution or Ca^{2+}-free Krebs solution. Figure 18 shows that the relaxant effect of progesterone was greater in the Ca^{2+}-free Krebs solution than in normal Krebs solution. The IC_{50} values are 24.97 ± 0.84 μM (n=7) and 14.64 ± 2.6 μM (n=9) in normal Krebs solution and Ca^{2+}-free Krebs solution, respectively ($P < 0.05$). The vessel tension induced by PDA is 9.8 ± 0.6 mN and 11.7 ± 0.4 mN in normal and Ca^{2+}-free Krebs solution, respectively.

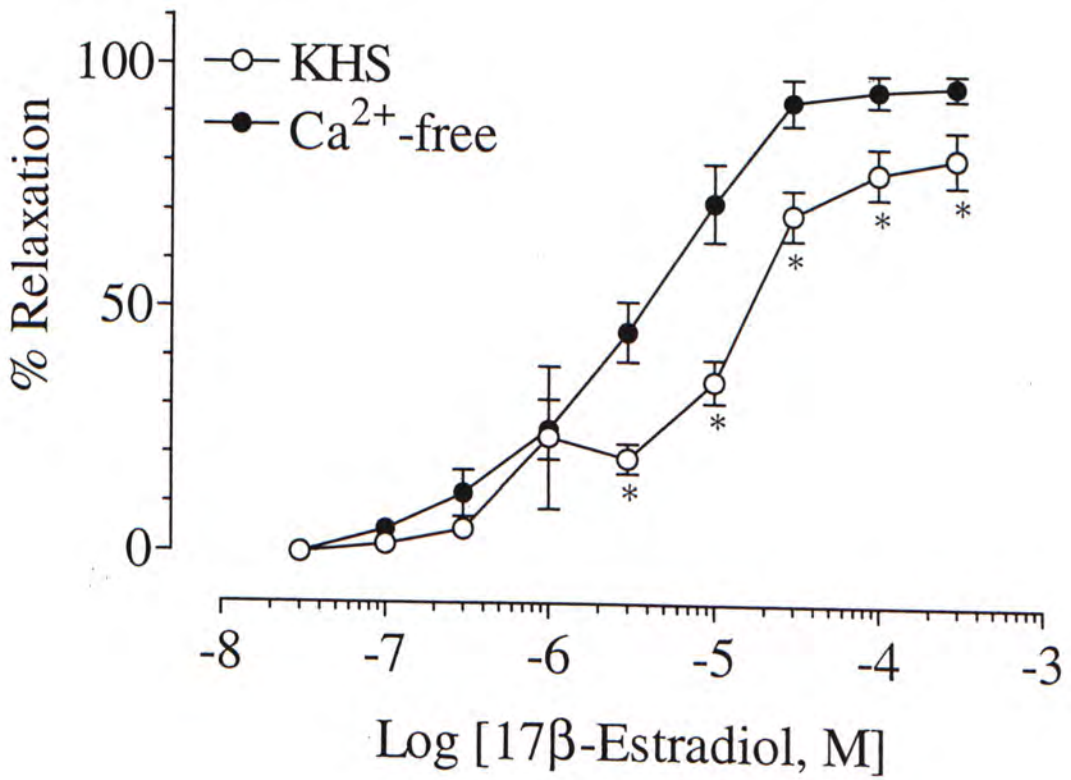

Figure 17

The effect of extracellular Ca^{2+} on 17β-estradiol-induced relaxation in PDA contraction (○, n=6 for normal Krebs solution; ●, n=6 for Ca^{2+}-free Krebs solution). The data are means ± S.E.M. of *n* experiments. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

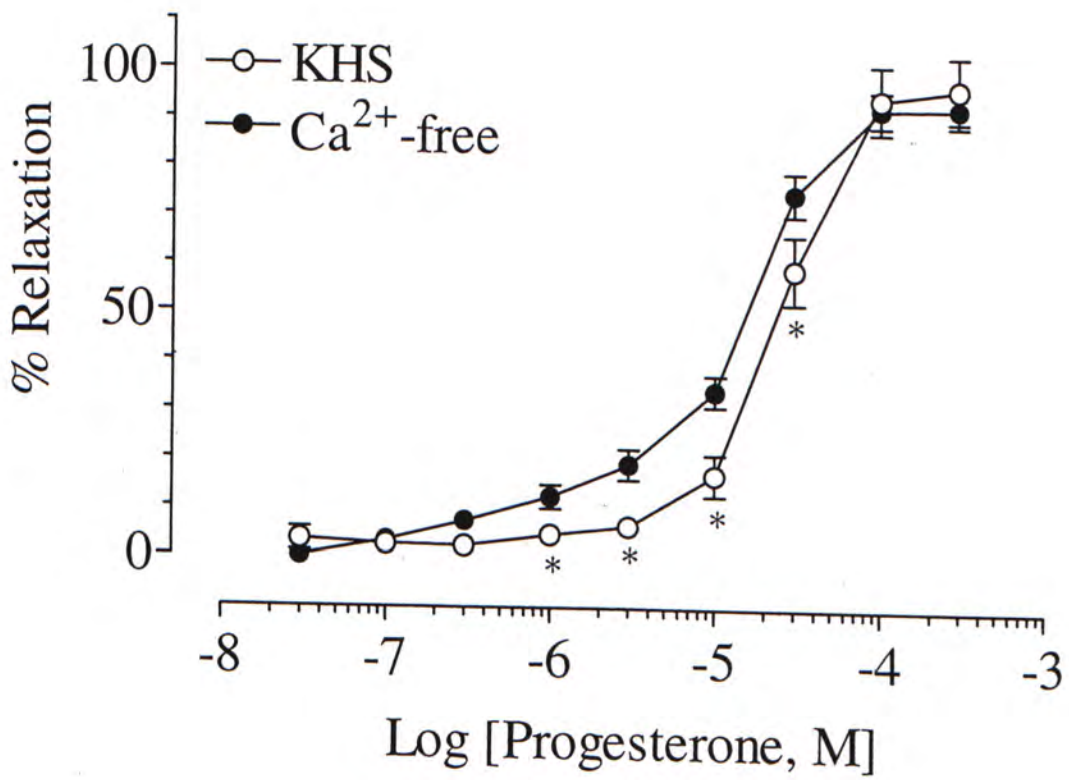

Figure 18

The effect of extracellular Ca^{2+} on progesterone-induced relaxation in PDA contraction (○, n=7 for normal Krebs solution; ●, n=9 for Ca^{2+}-free Krebs solution). The data are means ± S.E.M. of *n* experiments. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

3.5. Effects of β-adrenoceptor Agonists on 17β-Estradiol-induced Relaxations

3.5.1. Effect of isoproterenol on 17β-estradiol-induced relaxation

Isoproterenol induced concentration-dependent relaxation of phenylephrine-contracted rings with a pD_2 7.38 ± 0.21 (n=7) and this relaxation was significantly reduced in endothelium-denuded rings (pD_2: 5.59 ± 0.16, n=6, $P < 0.05$ compared with that in the presence of endothelium). Low concentrations of isoproterenol produced a slight relaxant response (3.3 ± 2.9 % and 7.6 ± 3.3 % relaxation, respectively in 1 and 3 nM isoproterenol, n=4) without an effect on the basal tone.

17β-Estradiol was previously shown to have an enhancing effect on the β-adrenoceptors-mediated vasorelaxant responses (Ferrer *et al.*, 1996). However, it is unknown whether 17β-estradiol and β-adrenoceptor agonists exert synergistic effects mutually. I therefore examined whether β-adrenoceptor agonists could also potentiate the relaxation induced by 17β-estradiol, in both endothelium-intact and -denuded rat mesenteric arteries. The traces in Figure 19b show that the relaxation induced by 17β-estradiol was enhanced after 60-minute pretreatment of low concentration of isoproterenol (1 nM), in comparison with the control (Figure 19a). Isoproterenol at two concentrations (1 and 3 nM) enhanced the 17β-estradiol-induced relaxation in endothelium-intact rings with the same potency (pD_2 values: 5.06 ± 0.06, n=8 for control; 5.97 ± 0.14, n=9 for 1 nM isoproterenol; 6.18 ± 0.28, n=5 for 3 nM isoproterenol, $P < 0.05$ compared with control, Figure 20), indicating that 1 nM is the concentration at which isoproterenol maximally enhanced the 17β-estradiol-induced relaxation.

In addition, the effect of isoproterenol (1 nM) was not different after the rings were exposed for 2.5 hours (pD_2 value: 5.85 ± 0.22, n=7, $P > 0.05$ compared with that obtained in 1-hr incubation time, Figure 21).

3.5.2. Role of endothelium/nitric oxide on the isoproterenol potentiation of 17β-estradiol-induced relaxation

Isoproterenol-induced relaxation induced both endothelium-dependent and –independent relaxation in rat mesenteric arteries (Huang & Kwok, 1997). It is possible that low concentration of isoproterenol may release some relaxing factors from the endothelial layer and subsequently enhanced the relaxant response to 17β-estradiol. Therefore, I examined the role of endothelium/nitric oxide in isoproterenol enhancing effect on 17β-estradiol-induced relaxation. It was found that this effect was absent in the endothelium-denuded rings (pD_2: 5.17 ± 0.07, n=7 for control and 5.31 ± 0.15, n=6 for 1 nM isoproterenol, $P > 0.05$, Figure 22).

To examine further the involvement of the endothelium, the effects of nitric oxide synthase inhibitors were investigated. Pretreatment with 30 μM L-NAME abolished the enhancing effect of isoproterenol completely (Figure 23 & 26). Furthermore, exposure to Rp8-cGMPS triethylamine (3 μM), a cyclic GMP-dependent protein kinase inhibitor, partially but significantly, inhibited the effect of isoproterenol (pD_2 value: 5.56 ± 0.13, n=6, $P < 0.05$ compared with the value obtained with isoproterenol alone, Figure 23 & 26). Both inhibitors had no effect on the basal tone or the maximal relaxation induced by 17β-estradiol (Figure 23).

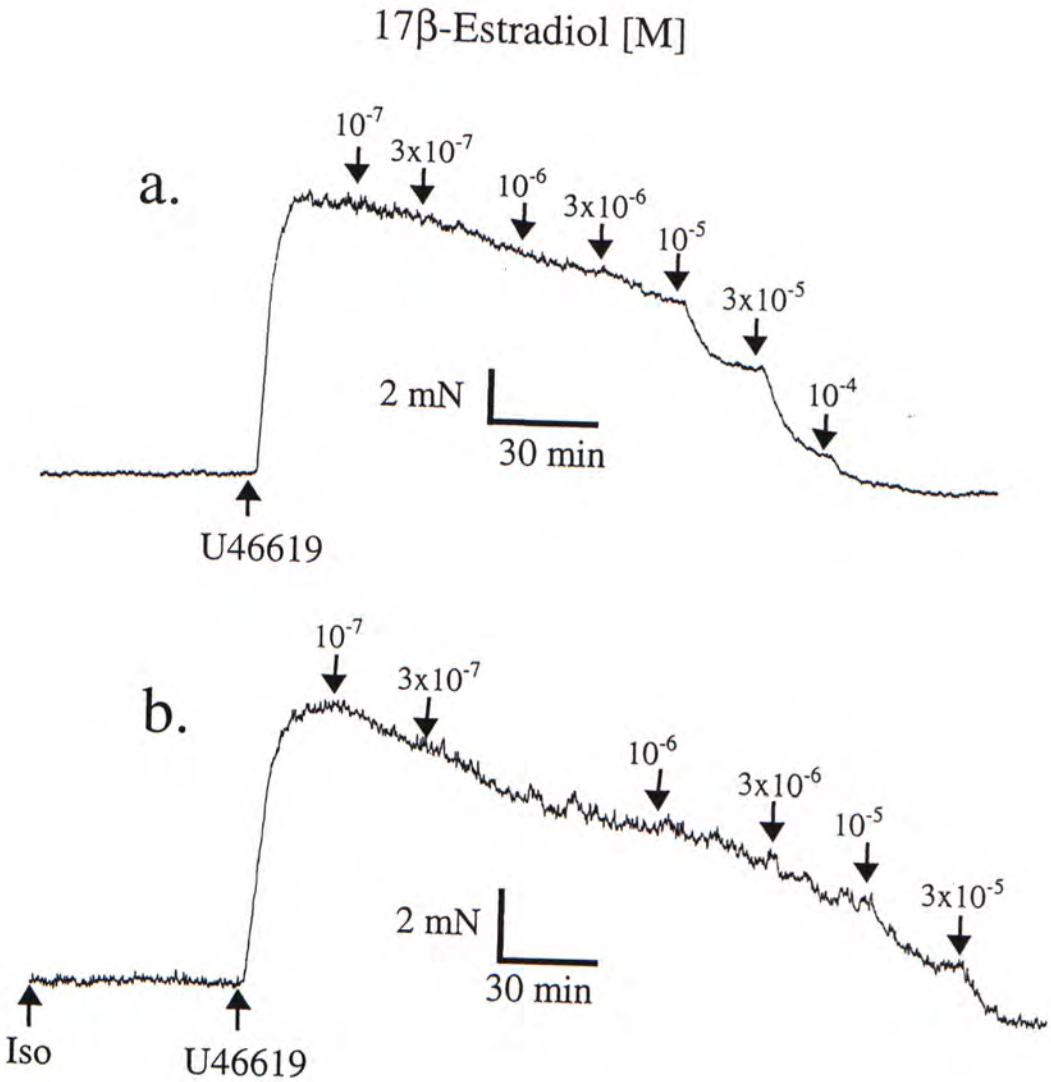

Figure 19

Representative records showing the concentration-dependent relaxant responses to 17β-estradiol in endothelium-intact rat mesenteric arteries in control (**a**) and in the ring pretreated with 10^{-9} M isoproterenol (Iso) for an hour (**b**).

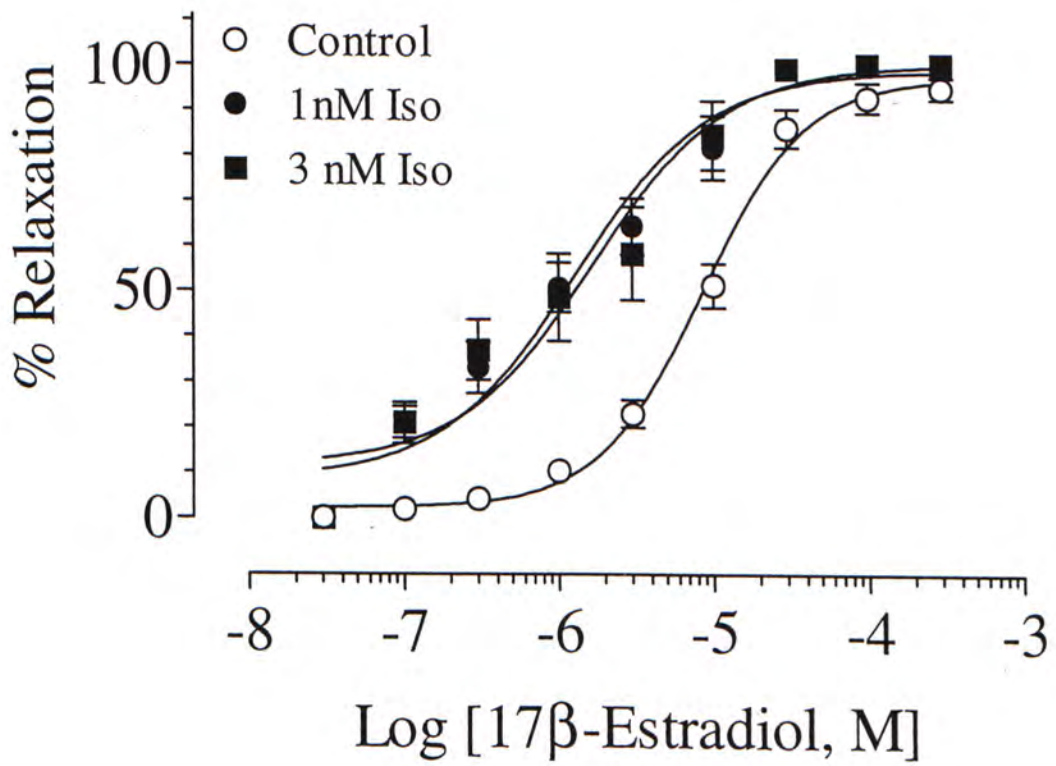

Figure 20

Concentration-response curves for 17β-estradiol-induced relaxation in endothelium-intact rings pretreated with isoproterenol (Iso) for 60 minutes (○, n=8 in control; ●, n=8 in 1 nM isoproterenol; and ■ n=5 in 3 nM isoproterenol). Data are means ± S.E.M. of *n* different rings.

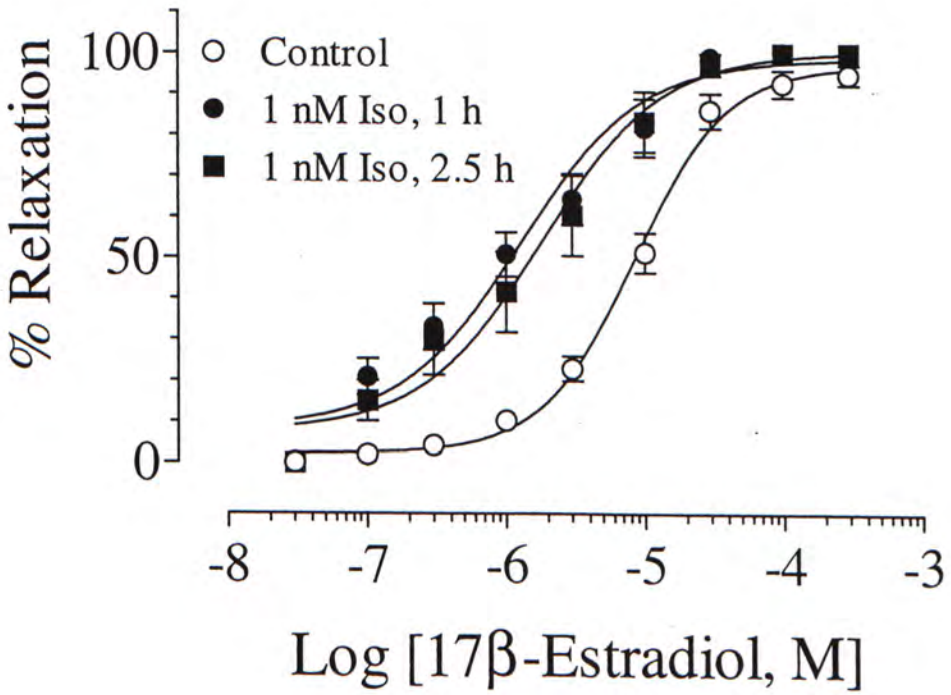

Figure 21

Concentration-response curves for 17β-estradiol-induced relaxation in rings pretreated with 1 nM isoproterenol (○, n=8 in control; ●, n=8 for 1 hour; and ■, n=7 for 2.5 hours). Data are means ± S.E.M. of *n* different rings.

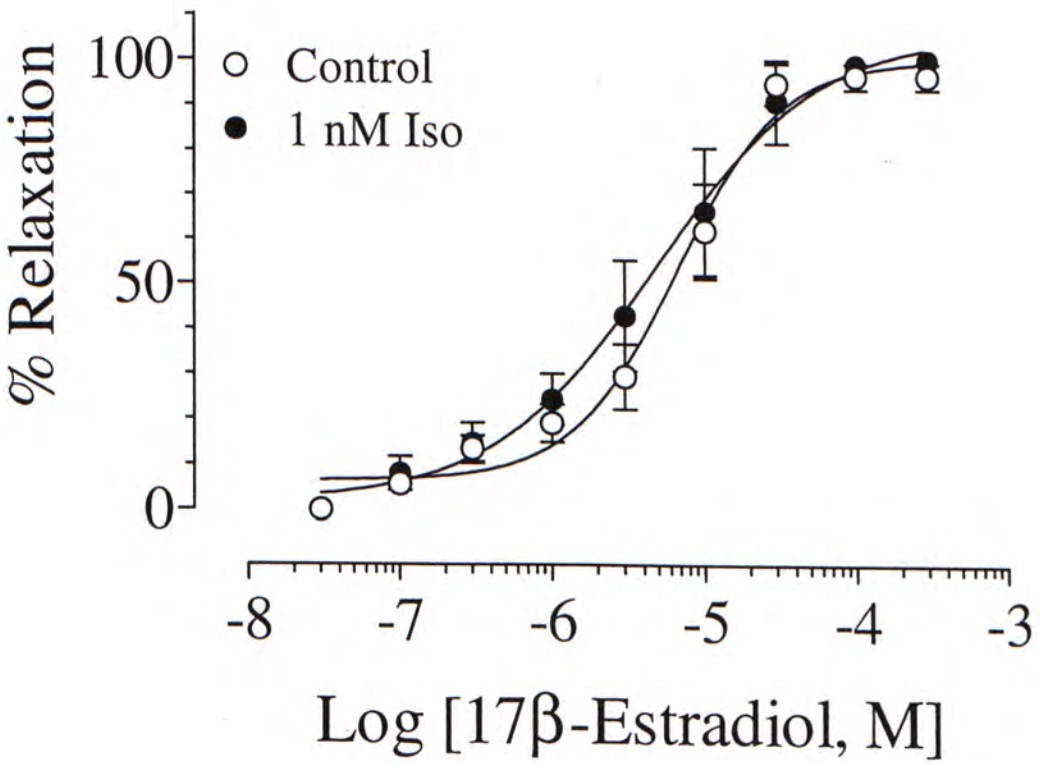

Figure 22

The concentration-response curves for 17β-estradiol-induced relaxation in the rat endothelium-denuded mesenteric artery rings pretreated with 1 nM isoproterenol (Iso) for 60 minutes (○, n=5 for control; ●, n=9 for isoproterenol). Data are means ± S.E.M. of *n* different rings.

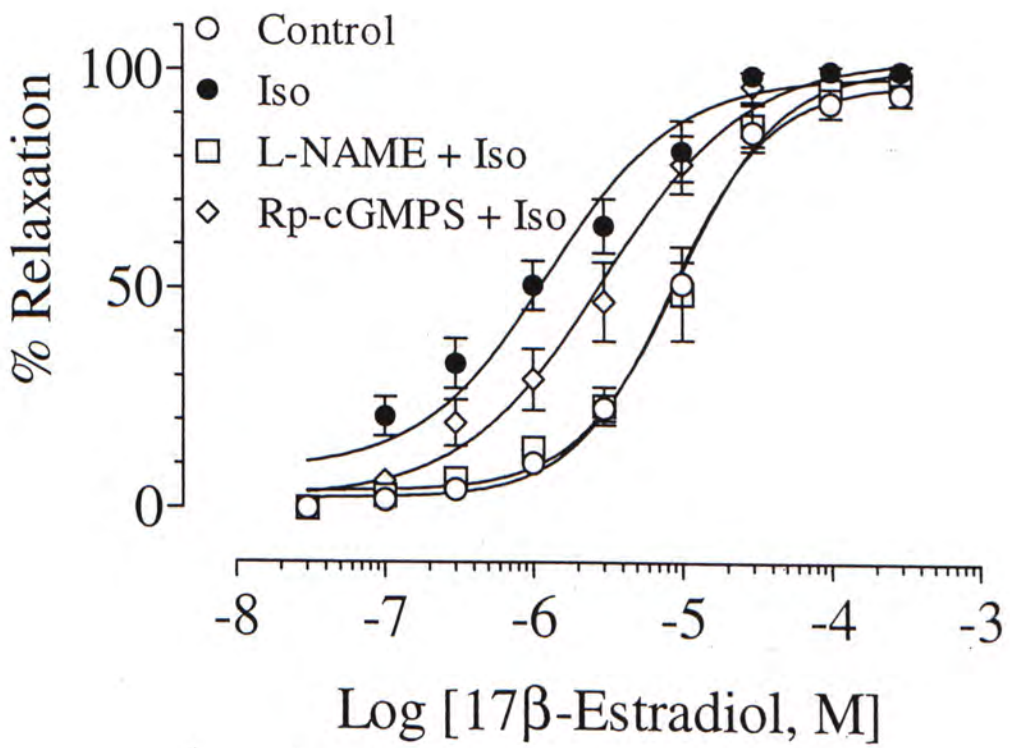

Figure 23

Effect of 30 μM L-NAME on isoproterenol (Iso) enhancement of 17β-estradiol-induced relaxation in endothelium-intact rings (○, n=8 in control; ●, n=8 in 1 nM isoproterenol; and □, n=5 in L-NAME plus 1 nM isoproterenol; ◇, n=5 in 3 μM Rp-cGMPS triethylamine plus 1 nM isoproterenol). Data are means ± S.E.M. of *n* different rings.

3.5.3. Role of cyclic AMP on isoproterenol-enhancement of 17β-estradiol-induced relaxation

Pretreatment with Rp-cAMPS triethylamine (3 μM, 30-min contact time), the cyclic AMP-dependent protein kinase inhibitor, significantly inhibited the effect of isoproterenol (pD_2 value: 5.25 ± 0.11, n=6, $P < 0.05$, compared with the value obtained with isoproterenol alone, Figure 24 & 26).

Pretreatment of endothelium-intact rings with 3 nM forskolin (30-min contact time) also amplified the relaxation induced 17β-estradiol (Figure 25 & 26). Co-treatment with forskolin (3 nM) and isoproterenol (1 nM) did not have additive effect (pD_2 values: 5.54 ± 0.17, n=5 for forskolin and 5.71 ± 0.09, n=6 for forskolin plus isoproterenol, $P > 0.05$, Figure 25). Neither Rp-cAMPS nor forskolin at concentrations used affected the basal tone.

3.5.4. Effects of β-adrenoceptor antagonists

Pretreatment with 3 μM propranolol (10 minutes before addition of isoproterenol), a non-selective β-adrenoceptor antagonist partially but significantly attenuated the effect of isoproterenol (Figure 27 & 30). ICI 118,551, a selective β_2 adrenoceptor, at 3 μM almost abolished the effect of isoproterenol (pD_2 values: 5.99 ± 0.14, n=8 for isoproterenol and 5.29 ± 0.14, n=6 for ICI 118,551 plus isoproterenol, $P < 0.05$, Figure 29 & 30). In contrast, 10 μM atenolol, a selective β_1-adrenoceptor antagonist, had no effect (Figure 28 & 30). None of three blocking agents influenced the basal tone or the relaxant response to 17β-estradiol (n=4-8, data not shown).

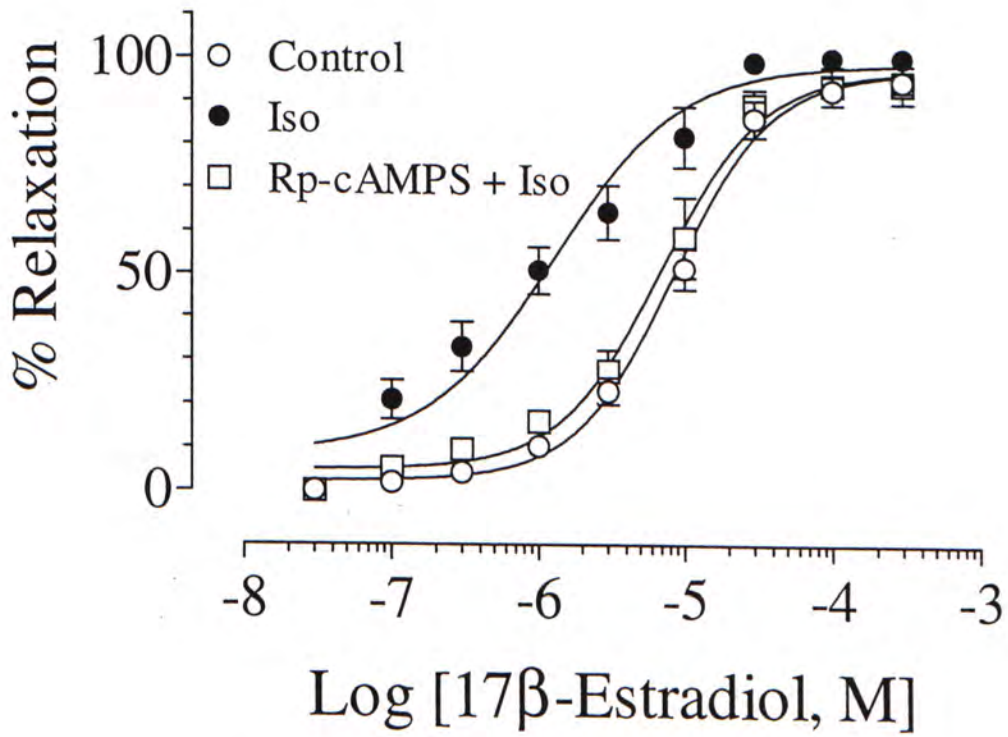

Figure 24

The effect of Rp-cAMPS triethylamine on isoproterenol enhancement of 17β-estradiol-induced relaxation in endothelium-intact rings (○, n=8 in control; ●, n=8 in 1 nM isoproterenol; and □, n=6 in Rp-cAMPS triethylamine). Data are means ± S.E.M. of *n* different rings.

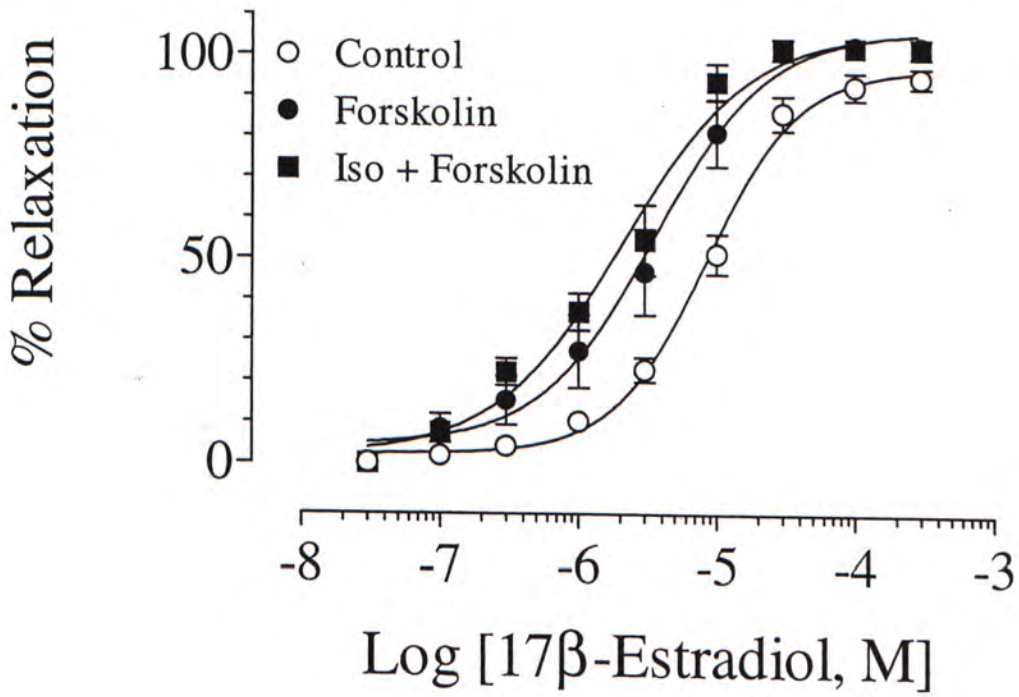

Figure 25

Effect of pretreatment with 3 nM forskolin on 17β-estradiol-induced relaxation in endothelium-intact rat mesenteric artery rings (○, n=8 in control; ●, n=5 in 3 nM forskolin; and ■, n=5 in 1 nM isoproterenol plus 3 nM forskolin). Data are means ± S.E.M. of *n* separate experiments.

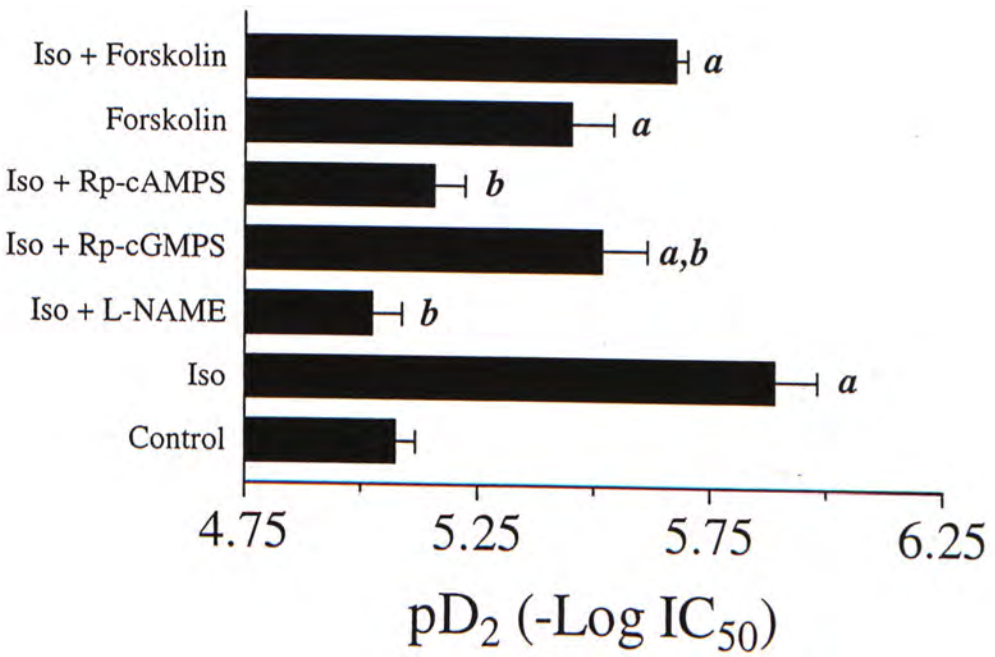

Figure 26

pD_2 values were calculated as the negative logarithm of the 17β-estradiol concentration that produces 50% the maximal relaxation following various pharmacological interventions in the isolated rat mesenteric artery rings. Data are means ± S.E.M. of *n* different rings. A significant difference ($P < 0.05$) is indicated by ***a*** between treatment and control groups and ***b*** between treatment and isoproterenol groups.

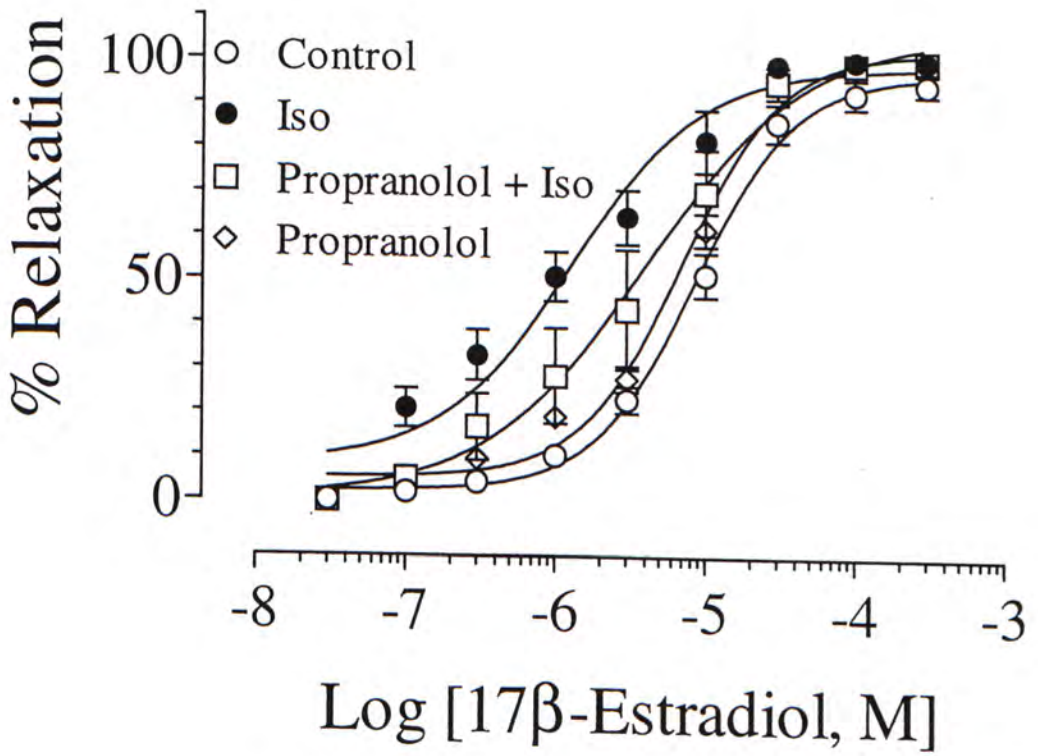

Figure 27

Effects of propranolol on isoproterenol enhancement of 17β-estradiol-induced relaxation in endothelium-intact rings. (a) The effect of 3 μM propranolol (○, n=8 in control; ●, n=8 in 1 nM isoproterenol; □, n=6 in propranolol plus isoproterenol; ◇, n=8 in propranolol). Data are means ± S.E.M. of *n* separate experiments.

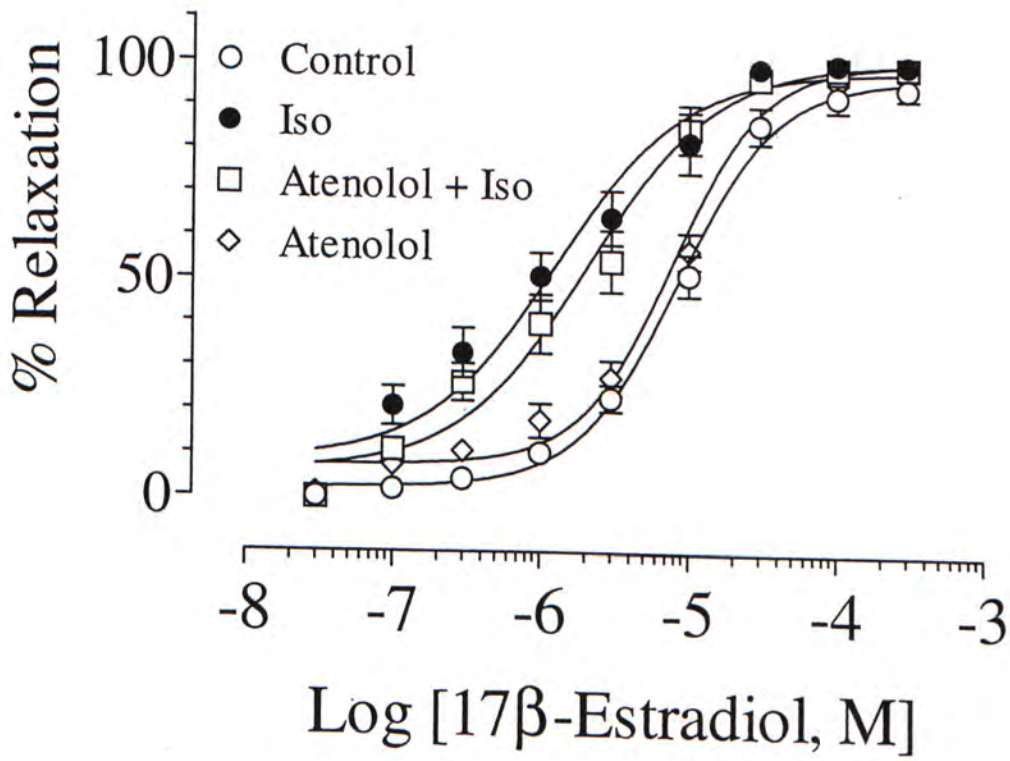

Figure 28

The effect of 10 μM atenolol on 17β-estradiol-induced contraction (○, n=8 in control; ●, n=8 in 1 nM isoproterenol; □, n=6 in atenolol plus isoproterenol; ◇, n=4 in atenolol). Data are means ± S.E.M. of *n* different rings.

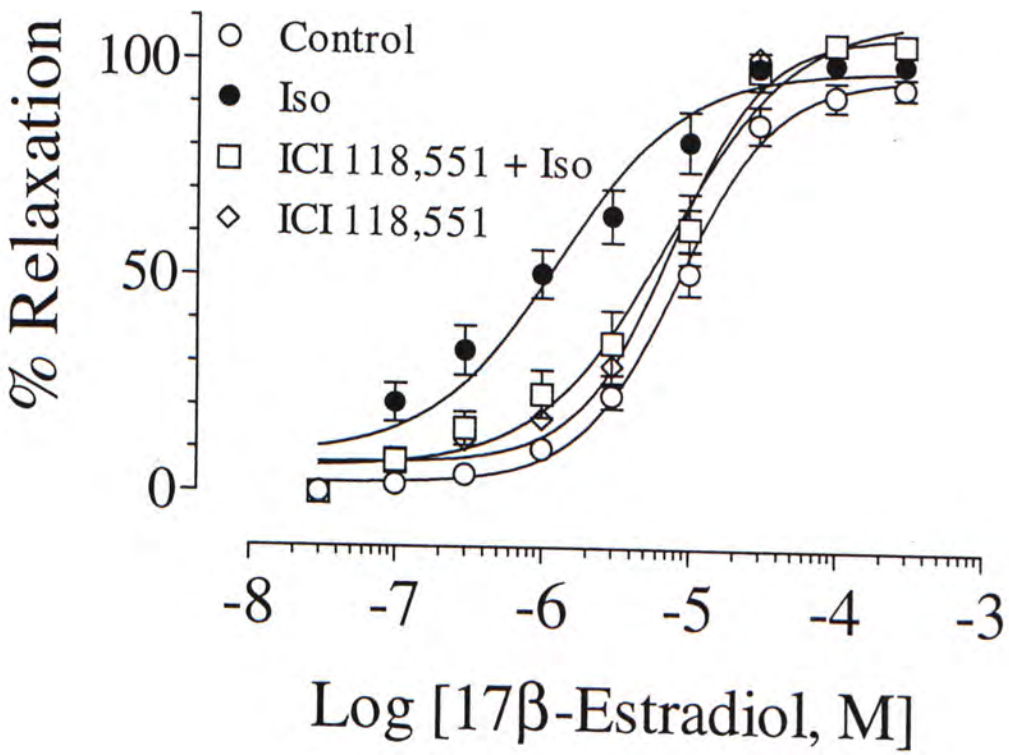

Figure 29

The effect of 3 μM ICI 118,551 on 17β-estradiol-induced relaxation (○, n=8 in control; ●, n=8 in 1 nM isoproterenol; □, n=6 in ICI 118,551 plus isoproterenol; ◇, n=5 in ICI 118,551). Data are means ± S.E.M. of *n* different rings.

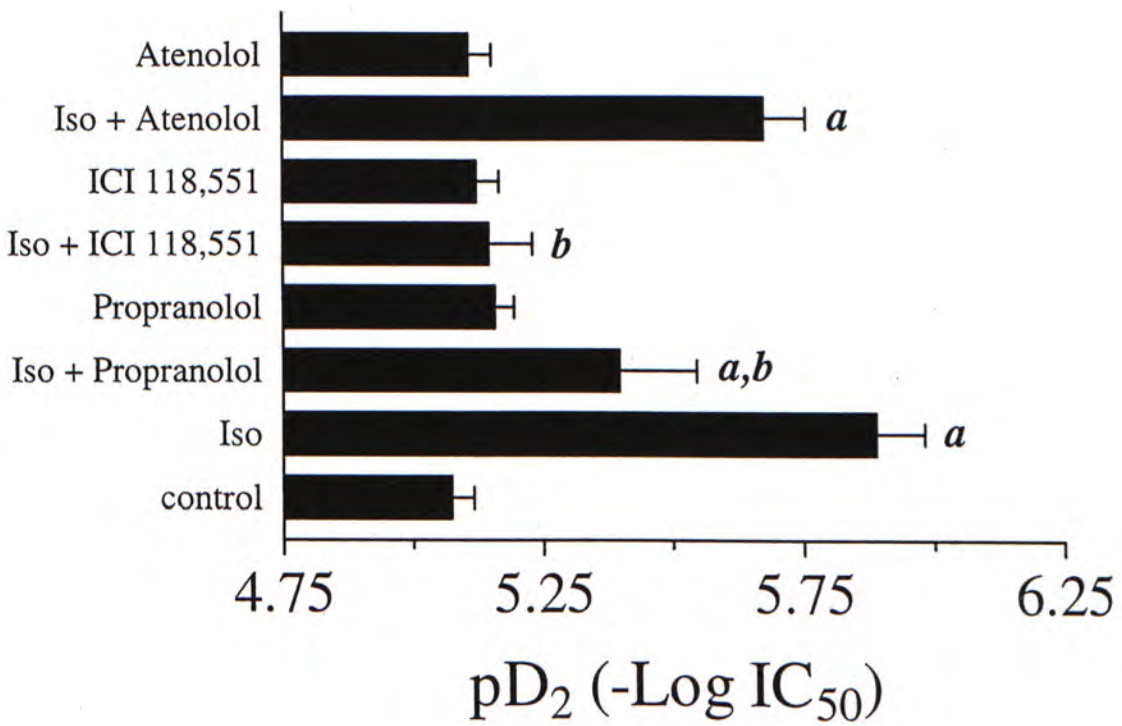

Figure 30

pD_2 values were calculated as the negative logarithm of the 17β-estradiol concentration that produces 50% the maximal relaxation following various pharmacological interventions in the isolated rat mesenteric artery rings. Data are means ± S.E.M. of *n* different rings. A significant difference ($P < 0.05$) is indicated by ***a*** between treatment and control groups and ***b*** between treatment and isoproterenol groups.

3.6. Effect of Physiological Concentration of 17β-Estradiol on β-adrenoceptor Agonists-induced Relaxations in Porcine Coronary Artery

Apart from the above-described experiments, another series of experiments were designed to examine whether 17β-estradiol would synergistically interact with β-adrenoceptor agonists. In these experiments, the isolated porcine coronary circumflex arteries were used.

3.6.1. Effect of 17β-estradiol on isoproterenol-induced relaxations

Acute exposure of 17β-estradiol (0.3 and 1 nM) for 20 minutes did not influence the relaxant effect of isoproterenol (pD_2 values: 7.47 ± 0.05 for control, n=6; 7.42 ± 0.17 for 0.3 nM 17β-E_2, n=6; 7.52 ± 0.11 for 1 nM 17β-E_2, n=6, $P > 0.05$, Figure 31a). Pretreatment of the same concentration of 17β-estradiol for 1 hour had a small effect on isoproterenol-induced relaxations (pD_2 values: 7.47 ± 0.1 for control, n=7; 7.62 ± 0.08 for 0.3 nM 17β-estradiol, n=6; 7.8 ± 0.05 for 1 nM 17β-estradiol, n=5, $P > 0.05$, Figure 31b).

3.6.2. Effect of 17β-estradiol on fenoterol-induced relaxations

The effect of 17β-estradiol on fenoterol (a β_2-adrenergic agonist)-induced relaxation was investigated. The concentration-response curve of fenoterol was unaffected following 20-minute pretreatment with two concentrations of 17β-estradiol (0.3 and 1 nM) ($P > 0.05$, Figure 33a). However, 1-hr incubation with 0.3 nM 17β-estradiol enhanced the fenoterol-induced relaxation (Figure 32), whilst neither 0.1 nor

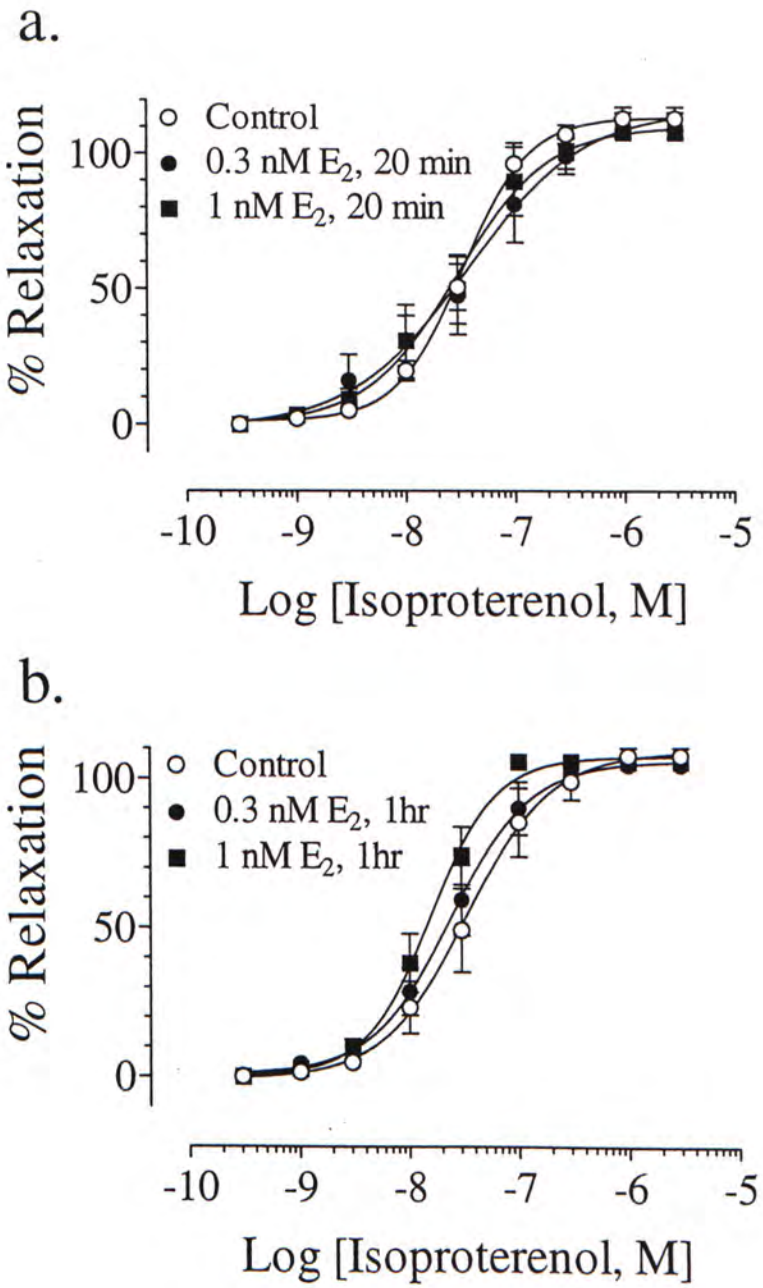

Figure 31

(a) Concentration-response curves for relaxation induced by isoproterenol after pretreatment with 17β-estradiol for 20 minutes in the endothelium-intact porcine coronary arteries (○, n=6 for control; ●, n=6 for 0.3 nM 17β-estradiol; ■, n=6 for 1 nM 17β-estradiol). **(b)** Concentration-response curves for isoproterenol-induced relaxation after exposure to 17β-estradiol for 1 hour (○, n=7 for control; ●, n=6 for 0.3 nM 17β-estradiol; ■, n=6 for 1 nM 17β-estradiol). Data are means ± S.E.M. of *n* different rings.

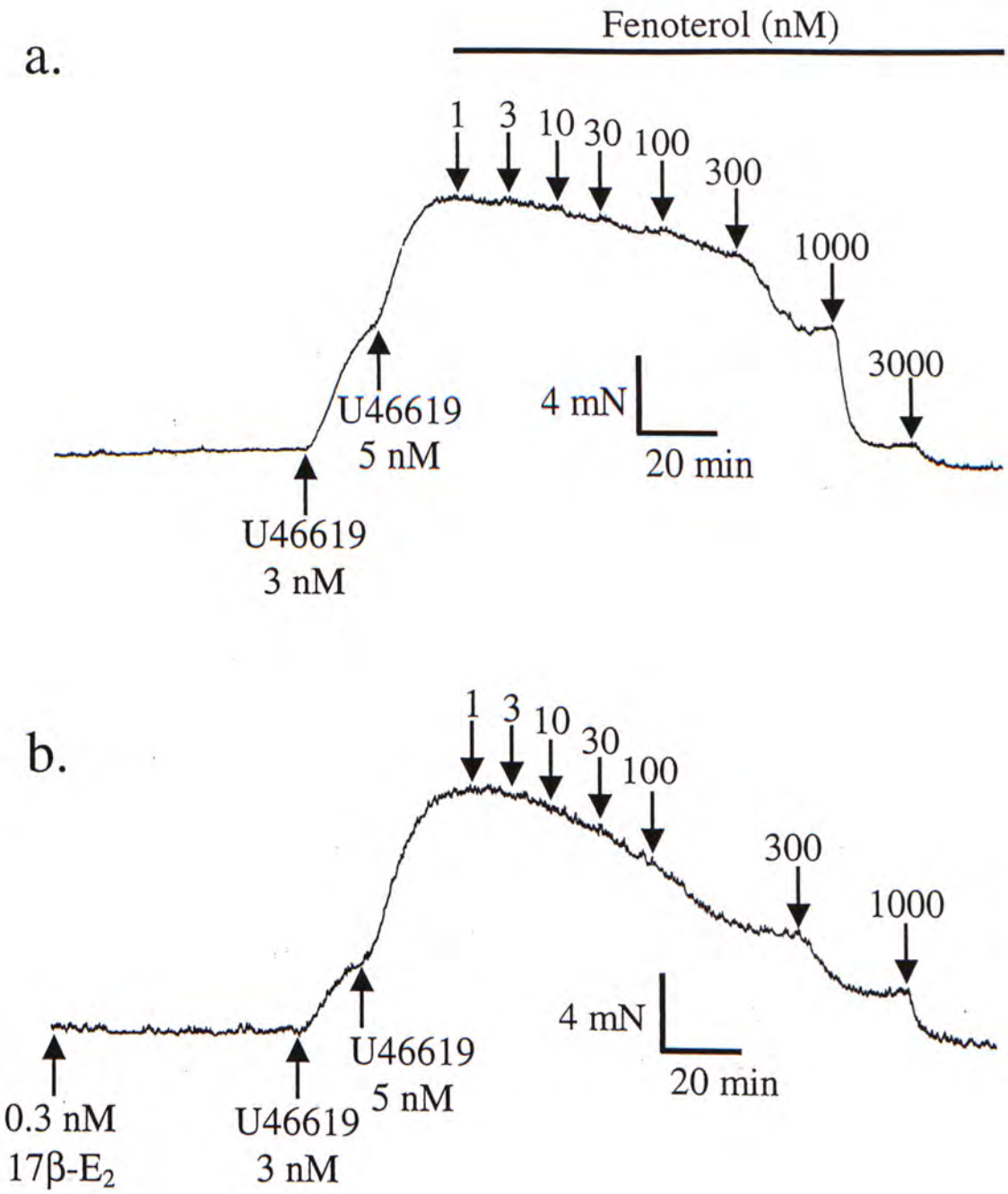

Figure 32

Representative records showing the concentration-dependent relaxant responses to fenoterol in endothelium-intact porcine coronary arteries in control (**a**) and in the ring pretreated with 0.3 nM 17β-estradiol for 1 hour (**b**).

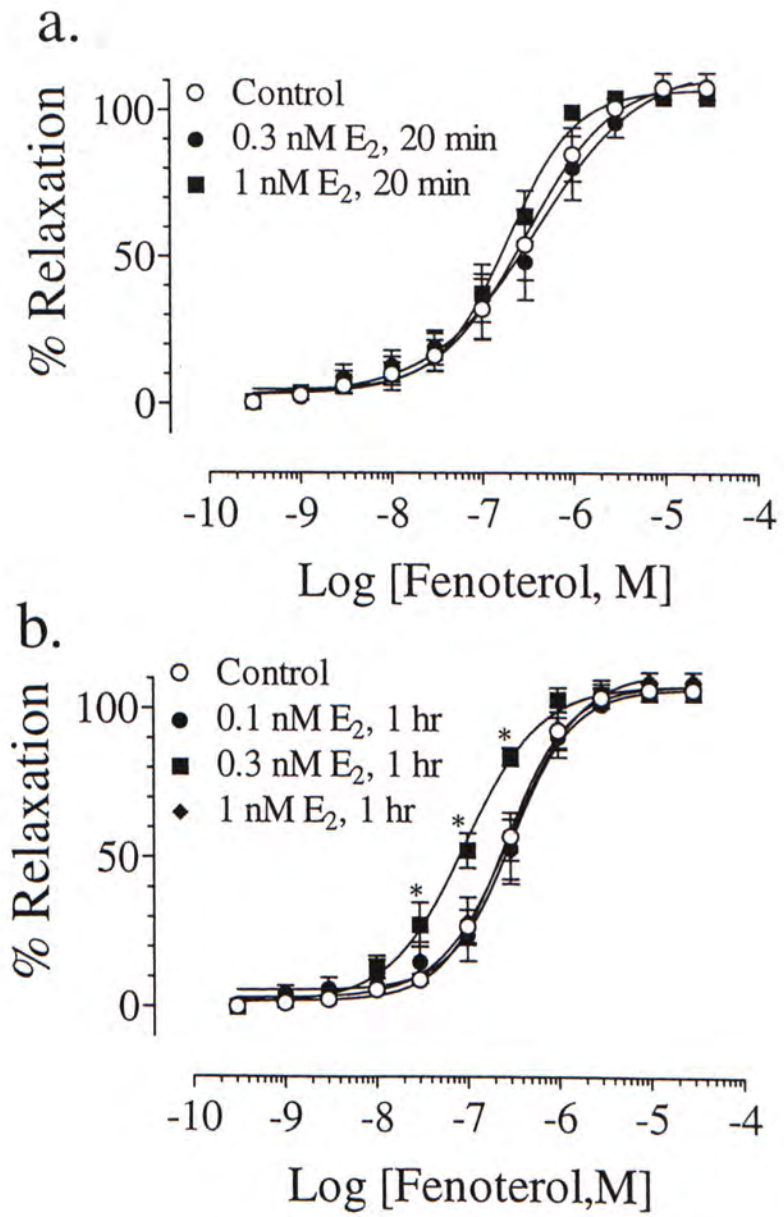

Figure 33

(a) Concentration-response curves for relaxation induced by fenoterol after pretreatment with 17β-estradiol for 20 minutes in the endothelium-intact porcine coronary arteries (○, n=8 for control; ●, n=6 for 0.3 nM 17β-estradiol; ■, n=5 for 1 nM 17β-estradiol). **(b)** Concentration-response curves for fenoterol-induced relaxation after exposure to 17β-estradiol for 1 hour (○, n=7 for control; ●, n=7 for 0.1 nM 17β-estradiol; ■, n=7 for 0.3 nM 17β-estradiol; ◆, n=8 for 1 nM 17β-estradiol). Data are means ± S.E.M. of *n* different rings. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

1 nM 17β-estradiol had such effect (pD_2 values: 6.58 ± 0.05 for control, n=7; 6.51 ± 0.07 for 0.1 nM, n=7; 7.02 ± 0.05 for 0.3 nM, n=7, $P < 0.05$ compared with control; 6.52 ± 0.07 for 1 nM, n=8, Figure 33b & 36).

Furthermore, the effect of tamoxifen was also examined. Figure 34 show that 10 μM tamoxifen abolished the effect of 17β-estradiol on fenoterol-induced relaxation. It is clear that the concentration-response curve for fenoterol was shifted towards the control following exposure to tamoxifen (pD_2 values: 6.58 ± 0.05 for control, n=7; 7.02 ± 0.05 for 0.3 nM 17β-estradiol, n=7, $P < 0.05$ compared with control; 6.55 ± 0.06 for 0.3 nM 17β-estradiol plus 10 μM tamoxifen, n=5; 6.28 ± 0.07 for 10 μM tamoxifen alone, n=5, Figure 35 & 36).

3.6.3. Effect of 17β-estradiol on dobutamine-induced relaxations

Figure 37a shows that 20-minute treatment with two concentrations of 17β-estradiol (0.3 and 1 nM) had no effect on dobutamine-induced relaxations. Similar pD_2 values calculated from three concentration-response curves are: 6.02 ± 0.09 for control, n=5; 5.98 ± 0.1 for 0.3 nM 17β-estradiol, n=5 and 6.09 ± 0.1 for 1 nM 17β-estradiol, n=5 ($P > 0.05$). On the other hand, 1-hr exposure with 17β-estradiol (0.1-1 nM) had slightly inhibitory effect on the dobutamine-induced relaxations. However, the inhibitory effect was not significant in 0.1 nM 17β-estradiol treatment ($P > 0.05$ when compared with control), whilst 0.3 and 1 nM 17β-estradiol had inhibitory effect ($P < 0.05$ when compared with control) (pD_2 values: 6.13 ± 0.2 for control, n=6; 5.64 ± 0.14 for 0.1 nM, n=5; 5.52 ± 0.08 for 0.3 nM, n=7; 5.77 ± 0.11 for 1 nM, n=7, Figure 37b).

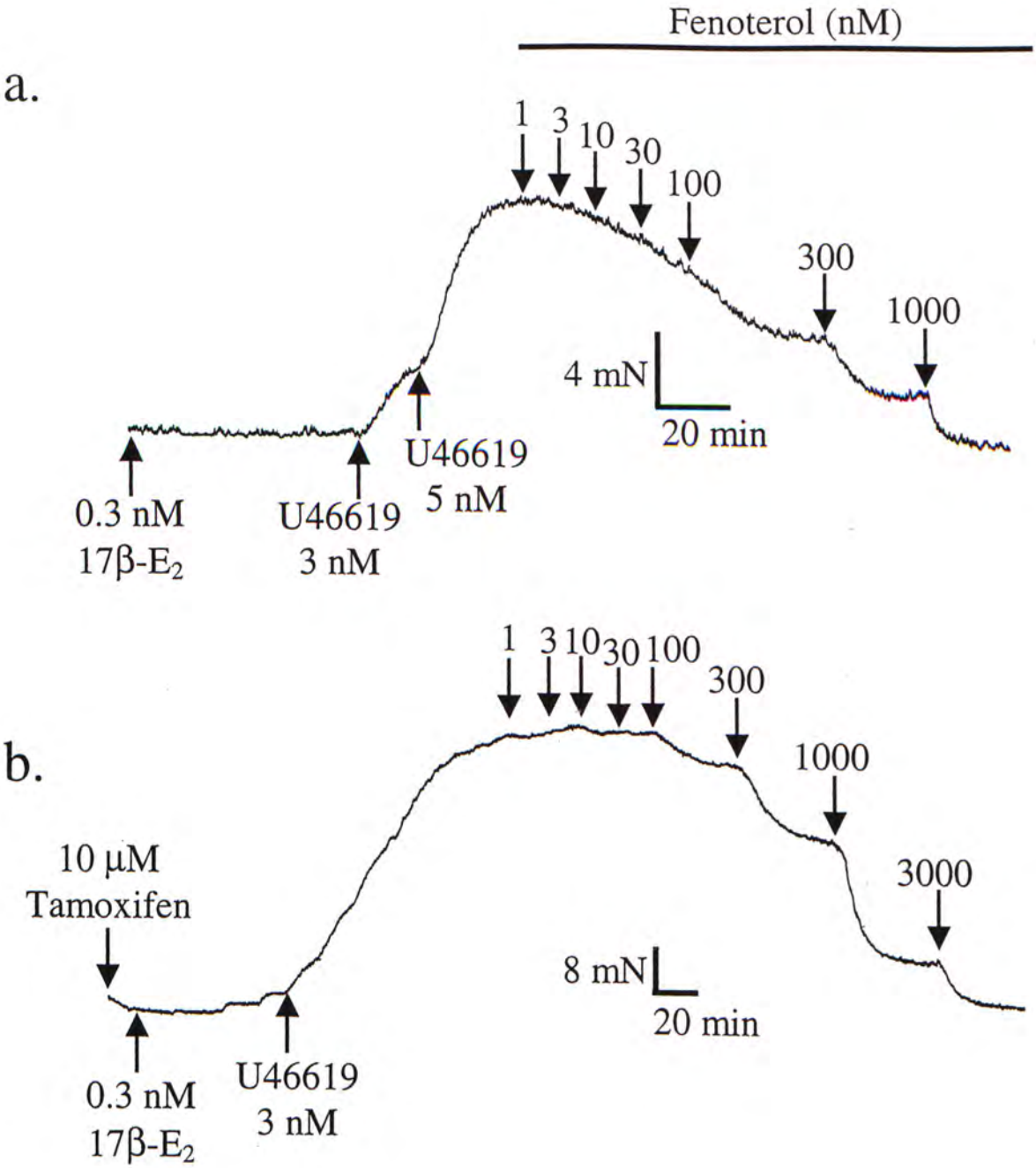

Figure 34

Representative records showing the effect of tamoxifen on the 17β-estradiol enhanced concentration-dependent relaxant responses to fenoterol in endothelium-intact porcine coronary arteries in the ring pretreated with 0.3 nM 17β-estradiol for 1 hr (**a**) and in the ring pretreated with 10 μM tamoxifen 10 minutes before adding 17β-estradiol (**b**).

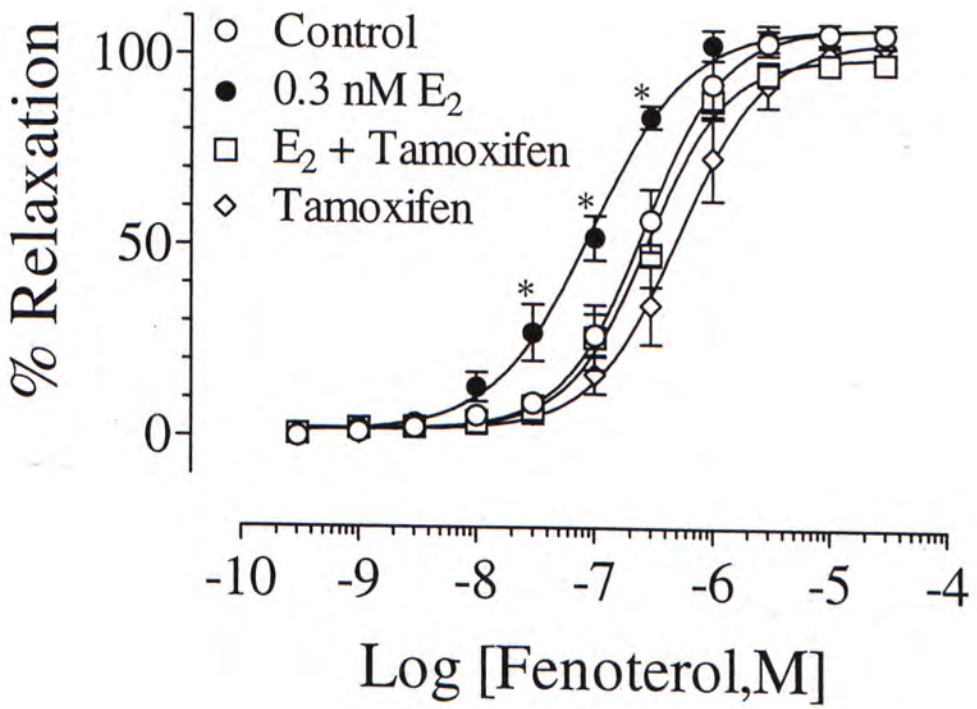

Figure 35

The effect of 17β-estradiol on fenoterol-induced relaxation for 1 hr (○, n=7 for control; ●, n=7 for 0.3 nM 17β-estradiol; □, n=5 for 0.3 nM 17β-estradiol plus 10 μM tamoxifen; ◇, n=5 for 10 μM tamoxifen). Data are means ± S.E.M. of *n* different rings. A significant difference between text and control values is indicated by asterisk* ($P < 0.05$).

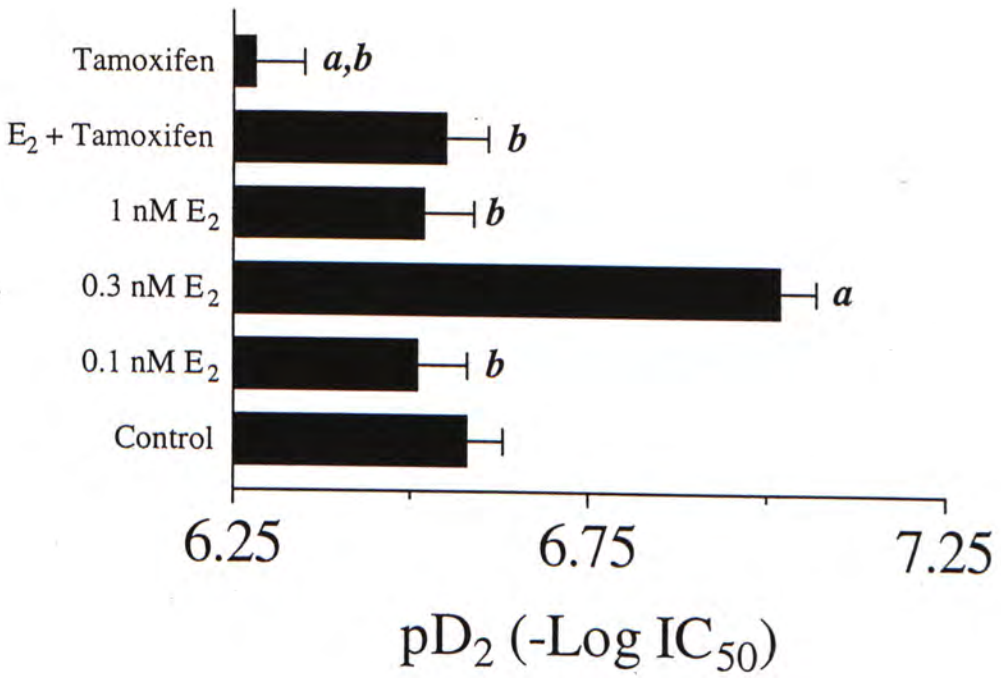

Figure 36

pD_2 values were calculated as the negative logarithm of the 17β-estradiol concentration that produces 50% the maximal relaxation following various pharmacological interventions in the isolated porcine coronary artery rings. Data are means ± S.E.M. of *n* different rings. A significant difference ($P < 0.05$) is indicated by ***a*** between treatment and control groups; ***b*** between treatment and 0.3 nM 17β-estradiol groups. The incubation time of all drugs is 1 hr.

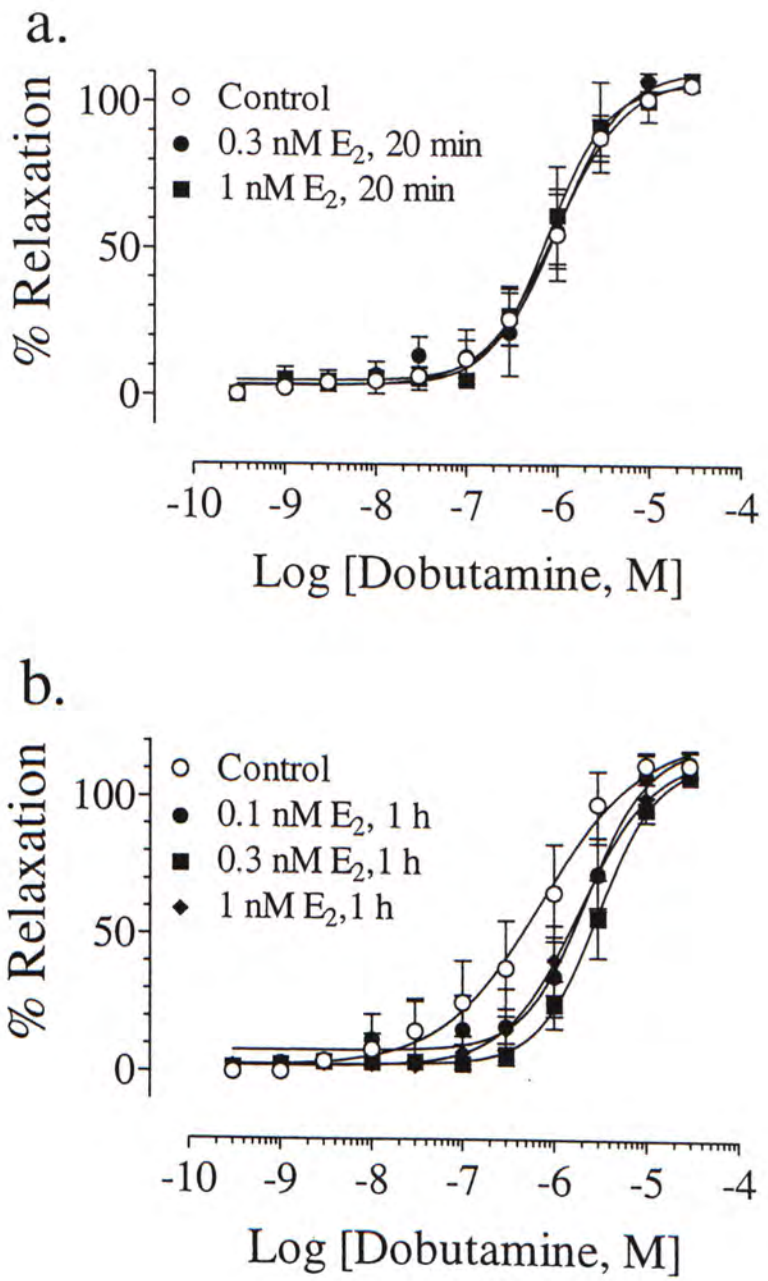

Figure 37

(a) Concentration-response curves for relaxation induced by dobutamine after pretreatment 17β-estradiol for 20 minutes in the endothelium-intact porcine coronary arteries (○, n=5 for control; ●, n=5 for 0.3 nM 17β-estradiol; ■, n=5 for 1 nM 17β-estradiol). **(b)** Concentration-response curves for dobutamine-induced relaxation after exposure to 17β-estradiol for 1 hr (○, n=6 for control; ●, n=5 for 0.1 nM 17β-estradiol; ■, n=7 for 0.3 nM 17β-estradiol; ◆, n=7 for 1 nM 17β-estradiol). Data are means ± S.E.M. of *n* different rings.

3.6.4. Effect of 17β-estradiol on IBMX-induced relaxations

The effect of 17β-estradiol on the relaxant response of 3-isobutyl-1-methylxathine (IBMX), a potent phosphodiesterase inhibitor, was also investigated. It was found that pretreatment with 17β-estradiol (0.1-1 nM) for 20 minutes did not affect the concentration-response curve for IBMX-induced relaxation (pD_2 values: 5.56 ± 0.05 for control, n=8; 5.85 ± 0.15 for 0.1 nM 17β-estradiol, n=5; 5.92 ± 0.05 for 0.3 nM 17β-estradiol, n=5; 5.82 ± 0.08 for 1 nM 17β-estradiol, $P > 0.05$, Figure 38a). Meanwhile, similar results were obtained in the 1-hr pretreatment of 17β-estradiol (pD_2 values: 5.92 ± 0.08 for control, n=5; 5.95 ± 0.08 for 0.3 nM 17β-estradiol, n=5; 6.06 ± 0.16 for 1 nM 17β-estradiol, n=5, $P > 0.05$, Figure 38b).

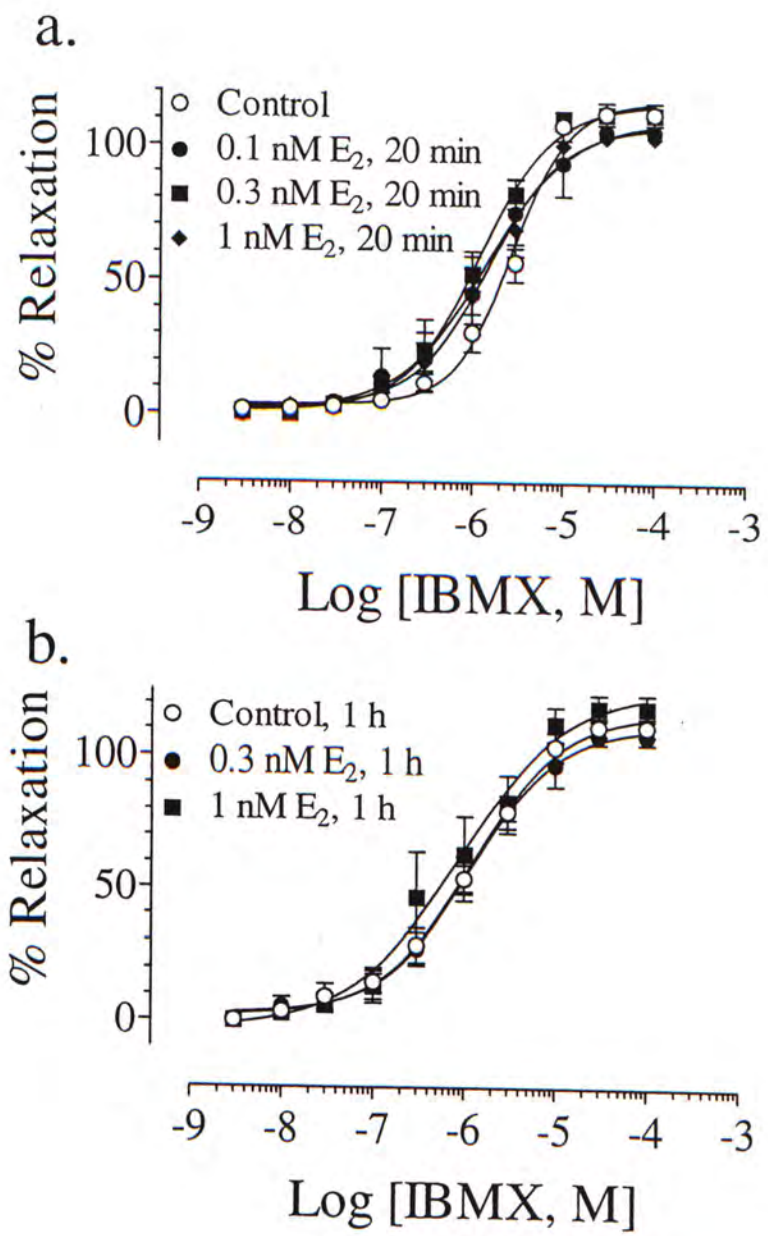

Figure 38

(a) The effect of 17β-estradiol on IBMX-induced relaxation following 20-minutes exposure in the endothelium-intact porcine coronary arteries (○, n=8 for control; ●, n=5 for 0.1 nM 17β-estradiol; ■, n=5 for 0.3 nM 17β-estradiol; ◆, n=5 for 1 nM 17β-estradiol). **(b)** The effect of 17β-estradiol on IBMX-induced relaxation after 1-hr exposure (○, n=5 for control; ●, n=5 for 0.3 nM 17β-estradiol; ■, n=5 for 1 nM 17β-estradiol). Data are means ± S.E.M. of *n* different rings.

3.7. Effect of Ovariectomy on the Vascular Reactivity

3.7.1. Effect of ovariectomy on the contractile activity of rat carotid arteries

3.7.1.1. Effect of ovariectomy on phenylephrine-induced contraction

In the endothelium-intact rat carotid arteries, phenylephrine (0.001-10 μM) produced concentration-dependent increase in vessel tension. There was no significant difference in phenylephrine-induced contraction among the control (n=6), estrogen-replaced (n=6, $P > 0.05$ when compared with the ovariectomized group), the ovariectomized group (n=6, $P > 0.05$ when compared with the control group) (Figure 39). Figure 40a shows that there was no difference in sensitivity of arterial rings to phenylephrine among the control (n=6), ovariectomized (n=6) and tamoxifen-replaced groups (n=4, $P > 0.05$ when compared with either the control or ovariectomized groups). Similar results were observed among the ovariectomized (n=6), ovariectomized estrogen-replaced (n=6, $P > 0.05$ when compared with co-treatment group), co-treatment of estrogen and tamoxifen group (n=6, $P > 0.05$ when compared with the ovariectomized group) (Figure 40b).

However, the results with phenylephrine-induced response were different in the endothelium-denuded rat carotid artery rings. The vasoconstriction effect of phenylephrine was significantly enhanced in ovariectomized rat carotid arteries.

Traces in Figure 41 show that lower concentrations (0.003-0.01 μM) of phenylephrine produced vasoconstriction in the ovariectomized rats (Figure 41b). In contrast, these two concentrations of phenylephrine did not induce measurable tension in the same ring preparation (Figure 41a). Figure 42 shows the summarized data presented in concentration-response curves for phenylephrine among three groups of rats. It is clear that ovariectomy enhanced the contractile response to phenylephrine at concentrations ranging between 10 nM and 10 μM ($P < 0.05$, between the control group, n=6, and the ovariectomized group, n=12). Estrogen replacement treatment partially but significantly reversed the effect of ovariectomy. The effect of estrogen replacement became significant in contraction induced by phenylephrine at concentrations higher than 100 nM (Figure 42). The statistical analysis was performed and presented between the ovariectomized group (n=12) and the estrogen-replaced group (n=6) (Figure 42).

I initially used tamoxifen pellet for the purpose of antagonizing the effect of chronic estrogen. Tamoxifen is a partial agonist on estrogen receptors, which could act as an antagonist in the presence of estrogen. However, to our surprise, tamoxifen pellet had the same effect as estrogen pellet on vascular contractility. Figure 43a shows that tamoxifen replacement therapy also partially reversed the enhancing effect of phenylephrine (0.3-10 μM) in carotid arteries from the ovariectomized rats. There

is statistical difference ($P < 0.05$) between the ovariectomized group (n=4) and the tamoxifen-replaced group (n=4). Furthermore, co-treatment with estrogen and tamoxifen caused additional reduction in the contractile response to phenylephrine (Figure 43b), suggesting that the chronic influence of 17β-estradiol and tamoxifen on vascular activity may be similar.

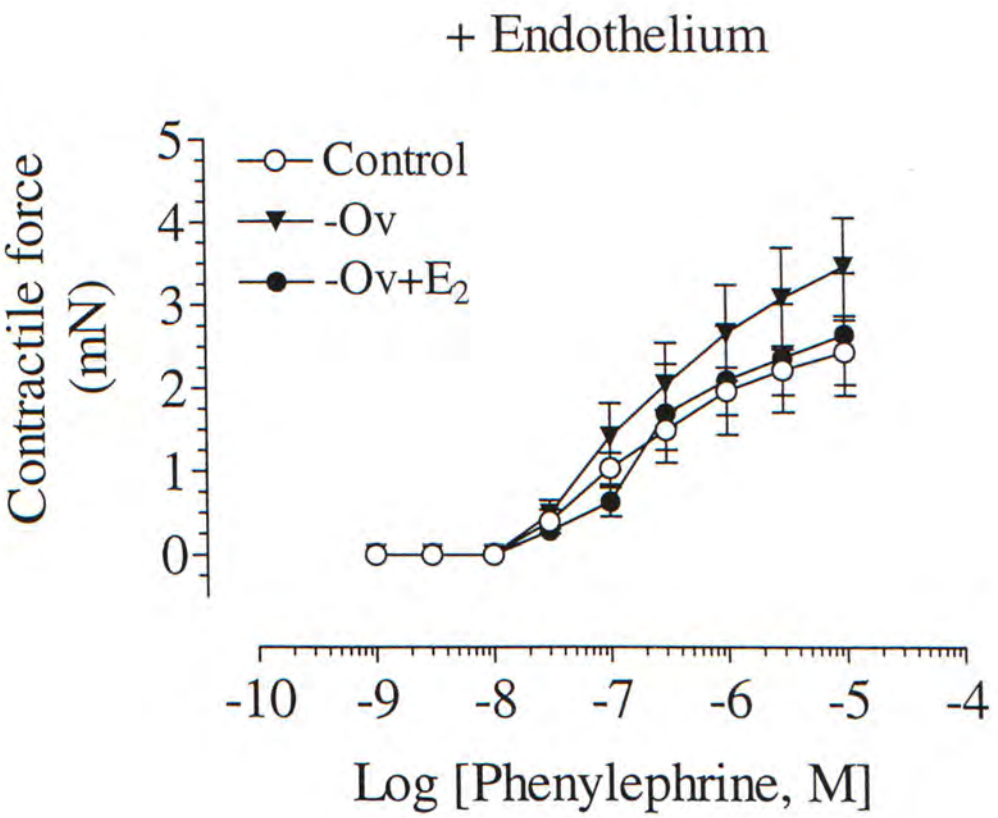

Figure 39

The effect of ovariectomy (-Ov) on the phenylephrine-induced contractions in the endothelium-intact rat carotid arteries. Concentration-response curves for phenylephrine in the control group (○, n=6), the ovariectomized group (▼, n=6) and the estrogen-replaced group (●, n=6). Data are means ± S.E.M. of *n* experiments.

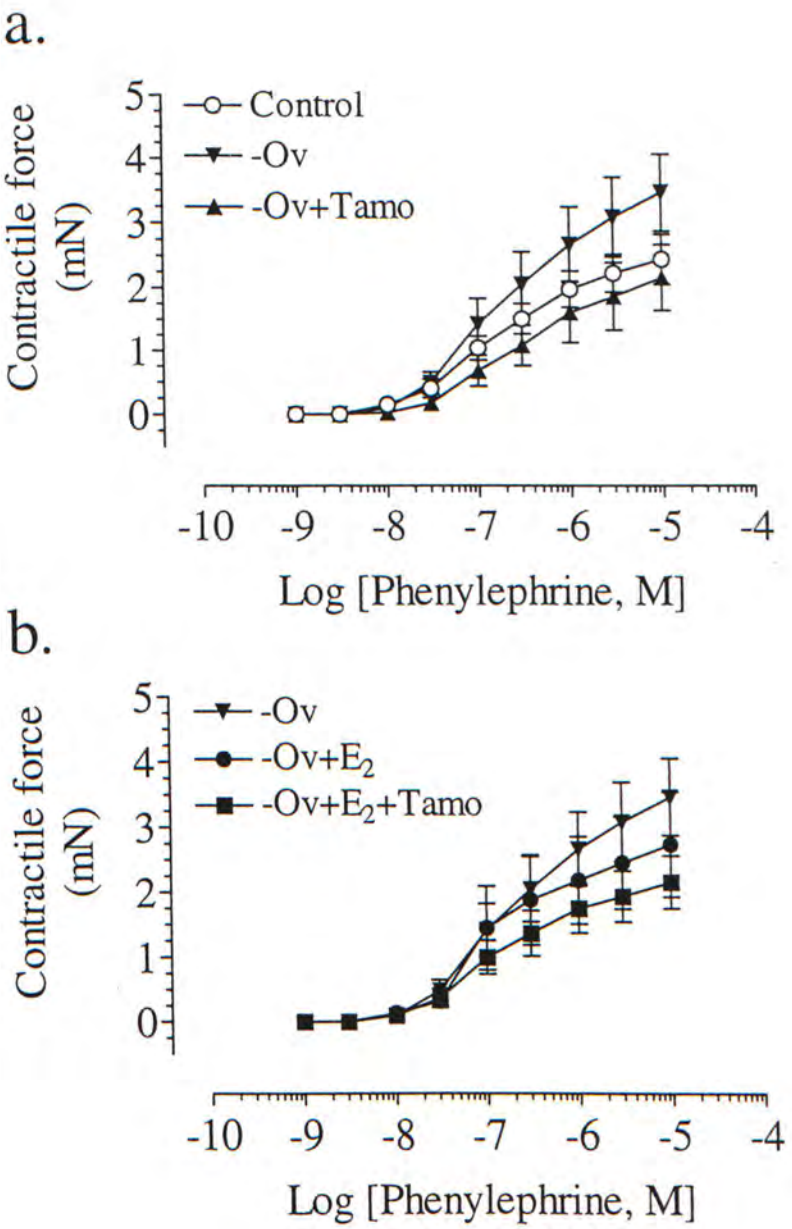

Figure 40

The effect of estrogen or/and tamoxifen replacement treatment on phenylephrine-induced contraction in endothelium-intact carotid arteries of the ovariectomized rats. **(a)** Concentration-response curves for phenylephrine in the control group (○, n=6), the ovariectomized group (▼, n=6) and the tamoxifen replaced group (▲, n=4). **(b)** Concentration-response curves for phenylephrine in the ovariectomized group (▼, n=6), the estrogen replaced group (●, n=6); the estrogen and tamoxifen replaced group (■, n=6). Data are means ± S.E.M. of *n* experiments.

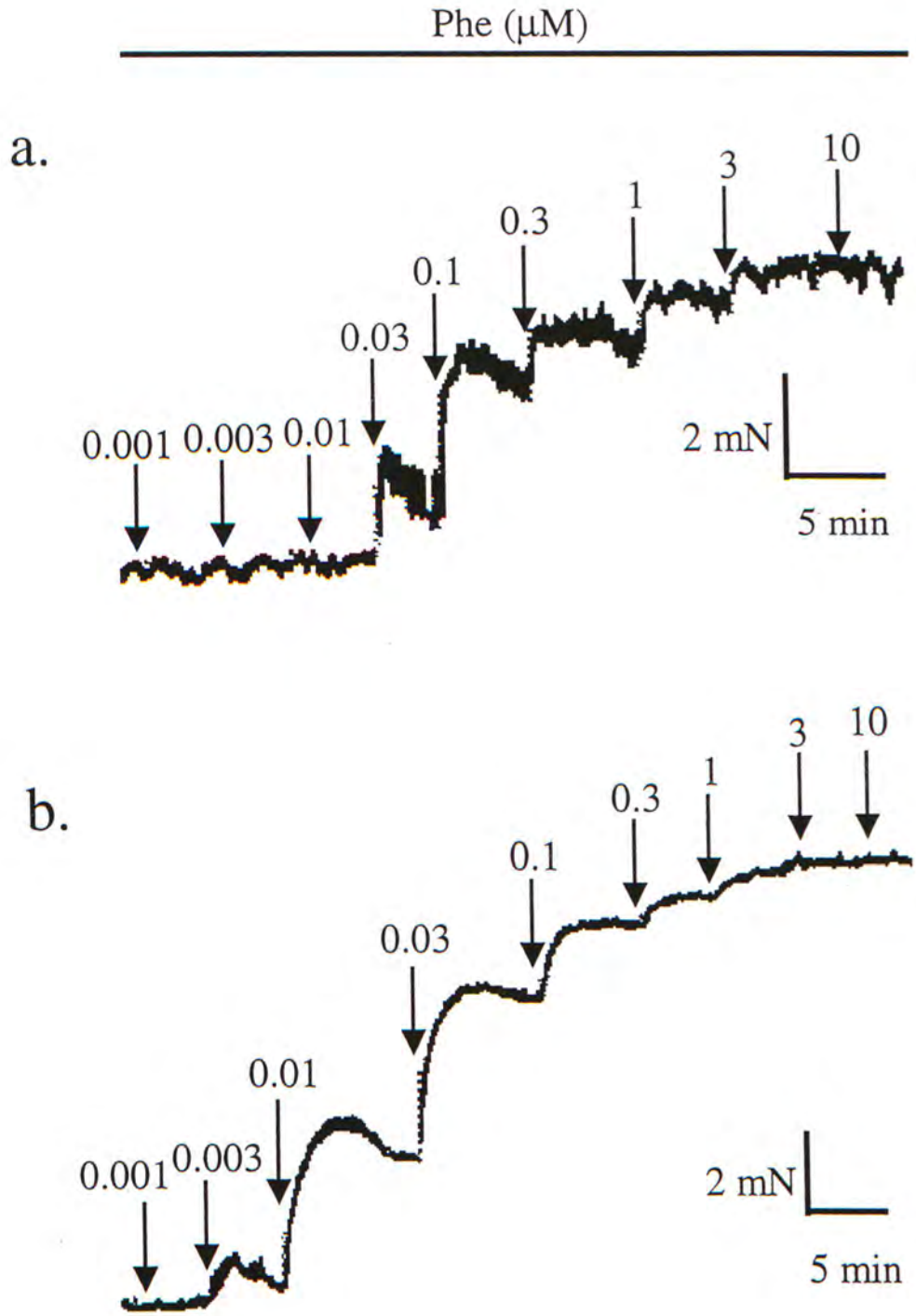

Figure 41

Representative traces showing the contractile response to phenylephrine in endothelium-denuded carotid arteries of the control rat (**a**) and the ovariectomized rat (**b**). Force and time were indicated by calibration bars.

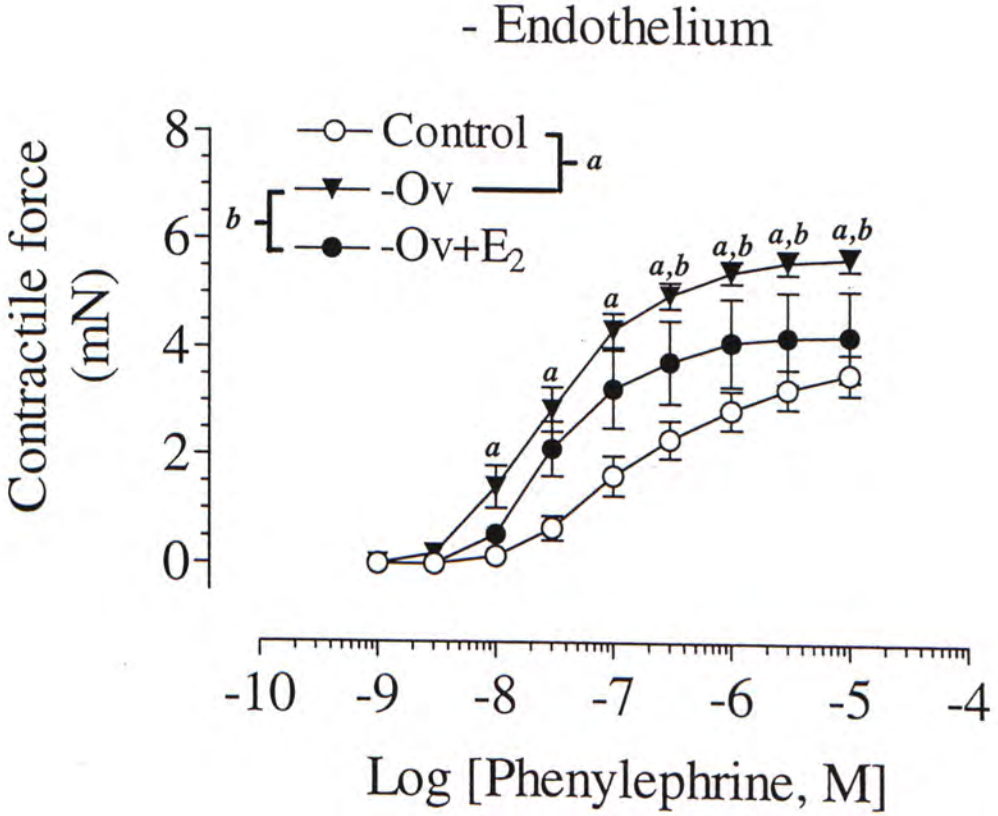

Figure 42

The effect of ovariectomy on the phenylephrine-induced contraction in isolated endothelium-denuded rat carotid arteries. Concentration-response curves for phenylephrine in the control group (○, n=6), the ovariectomized group (▼, n=12) and the estrogen-replaced group (●, n=6). Statistical difference ($P < 0.05$) was indicated as ***a*** between the control and ovariectomized groups; ***b*** between the ovariectomized and estrogen-replaced groups. Data are means ± S.E.M. of *n* experiments.

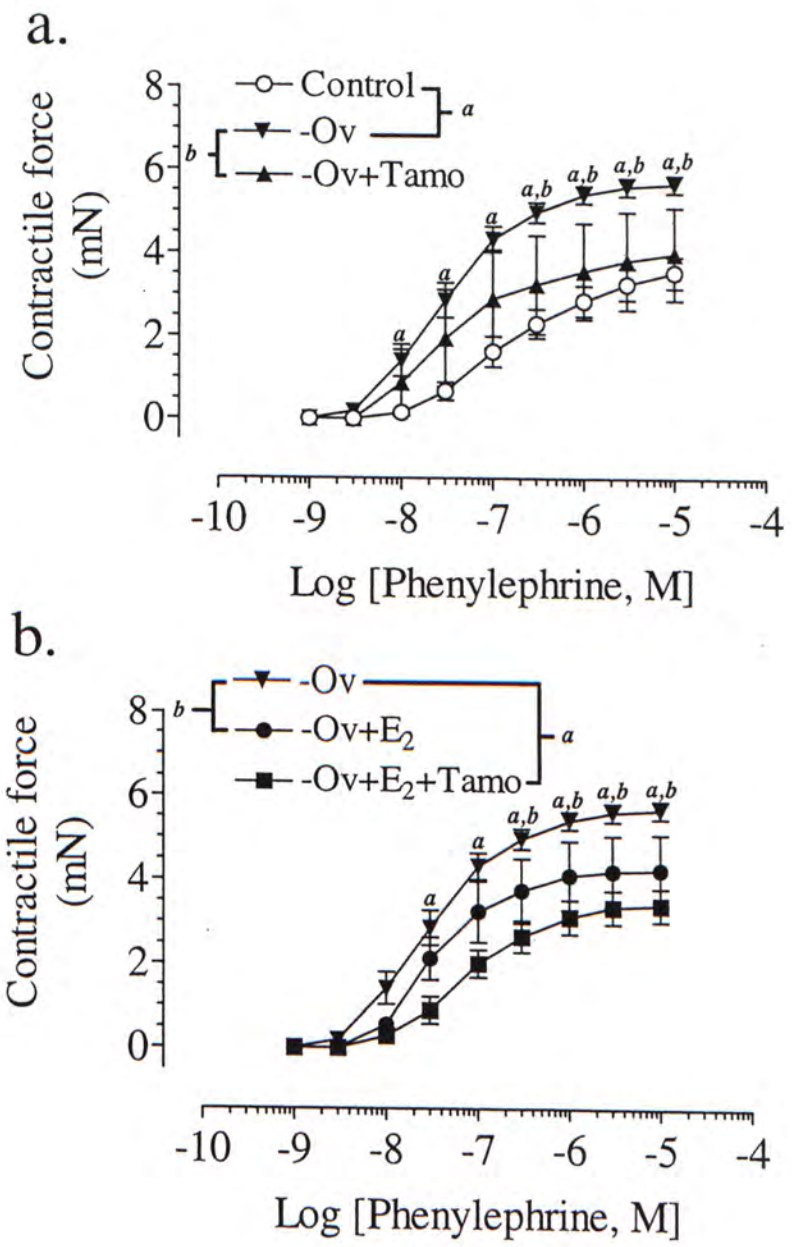

Figure 43

The effect of estrogen or/and tamoxifen replacement treatment on phenylephrine-induced contraction in endothelium-denuded carotid arteries from ovariectomized rats. **(a)** Concentration-response curves for phenylephrine in the control group (○, n=6), the ovariectomized group (▼, n=12), and the tamoxifen replaced group (▲, n=4). The statistical difference ($P < 0.05$) was indicated as ***a*** between control and ovariectomized groups; ***b*** between the ovariectomized and tamoxifen-replaced groups. **(b)** Concentration-response curves for phenylephrine in the estrogen replaced group (●, n=6), the ovariectomized group (▼, n=12), and estrogen and tamoxifen replaced group (■, n=6). Statistical difference ($P < 0.05$) was indicated as ***a*** in between the ovariectomized and tamoxifen-replaced group; ***b*** between the ovariectomized and tamoxifen-replaced groups. Data are means ± S.E.M. of *n* experiments.

3.7.1.2. Effect of ovariectomy on U46619-induced contraction

In the endothelium-intact rat carotid arteries, the thromboxane A_2 analogue, U46619 (1-300 nM) produced the contractile responses that were greater than that induced by phenylephrine. Ovariectomy did not affect the U46619-induced contraction (Figure 44). There is no significant difference among the control (n=6), ovariectomized (n=6) and ovariectomized, estrogen-replaced groups (n=6) ($P > 0.05$, Figure 44). Figure 45a shows that tamoxifen replacement therapy had a slight inhibitory effect on U46619 (30 nM)-induced contraction in the ovariectomized rats. No significant difference can be detected in other concentrations used for U46619 between the control and ovariectomized groups ($P > 0.05$, Figure 45a). Besides, co-replacement therapy with estrogen and tamoxifen (n=6) did not alter the contractile response to U46619 ($P > 0.05$, among the ovariectomized, estrogen-replaced and co-treatment groups ($P > 0.05$, Figure 45b).

On the other hand, the vasoconstricting responses to U46619 in the endothelium-denuded carotid arteries were different from those in the endothelium-intact rings even though the vessel tension developed by U46619 were similar (Figure 44 & Figure 46). Figure 46 shows the concentration-response curves for U46619-induced contraction in three treatment groups. It is clear that ovariectomy significantly potentiated U46619-mediated contraction ($P < 0.05$ between the control, n=6 and ovariectomized groups, n=6). Estrogen replacement treatment abolished this

enhancing effect of ovariectomy (Figure 46). Statistical difference in concentration-response curves for U46619 is presented between the ovariectomized group (n=6) and ovariectomized, estrogen-replaced groups (n=6) ($P < 0.05$, Figure 46).

Similar to the effect of tamoxifen pellet on phenylephrine-induced contraction, tamoxifen replacement treatment also fully reversed the enhancing effect of ovariectomy on vessel tone and this effect became apparent when the concentration of U46619 was greater than 10 nM ($P < 0.05$, n=5-6, Figure 47a). Figure 47b shows that co-treatment with estrogen and tamoxifen did not produce additive effect on U46619-inudced contraction in the carotid arteries prepared from the ovariectomized rats (n=6, $P > 0.05$ compared with estrogen replacement group).

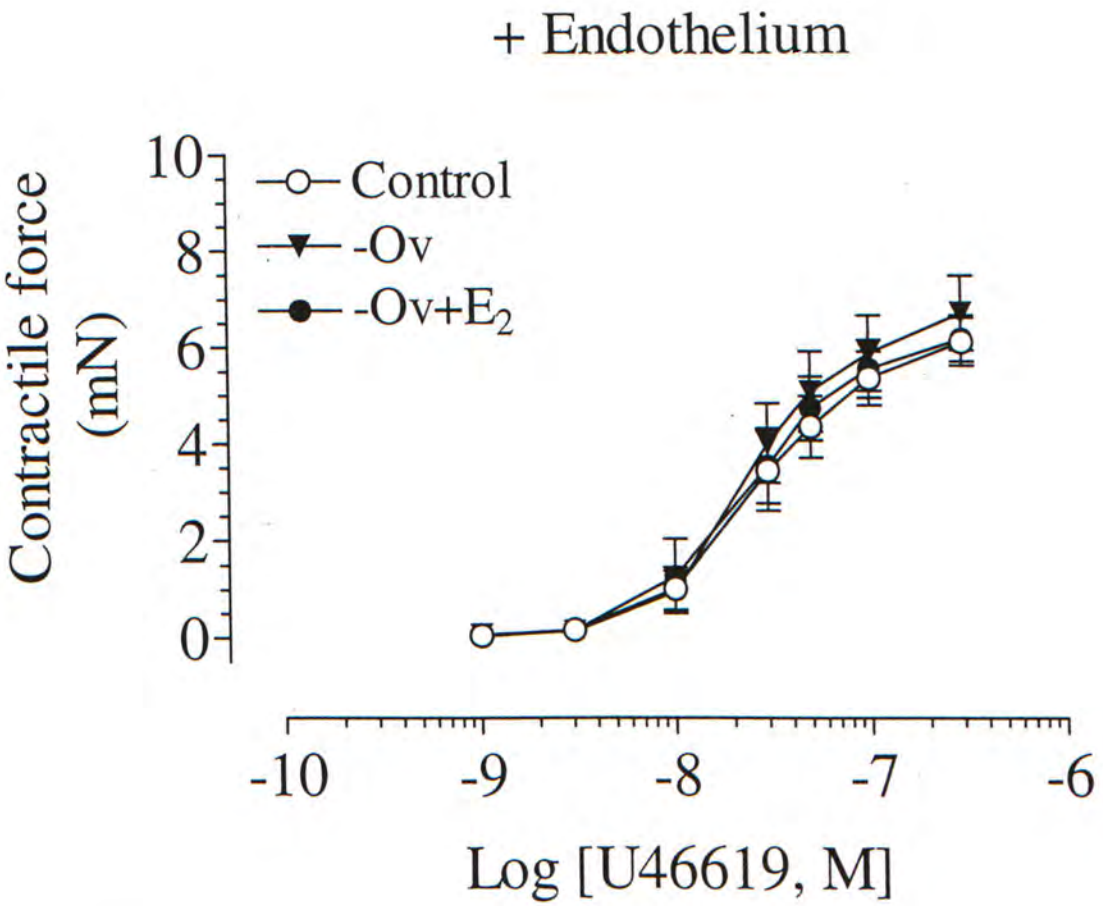

Figure 44

The effect of ovariectomy on the U46619-induced contraction in endothelium-intact rat carotid arteries. Concentration-response curves for U46619 in the control group (○, n=6), the ovariectomized group (▼, n=6) and the estrogen-replaced group (●, n=6). Data are means ± S.E.M. of *n* experiments.

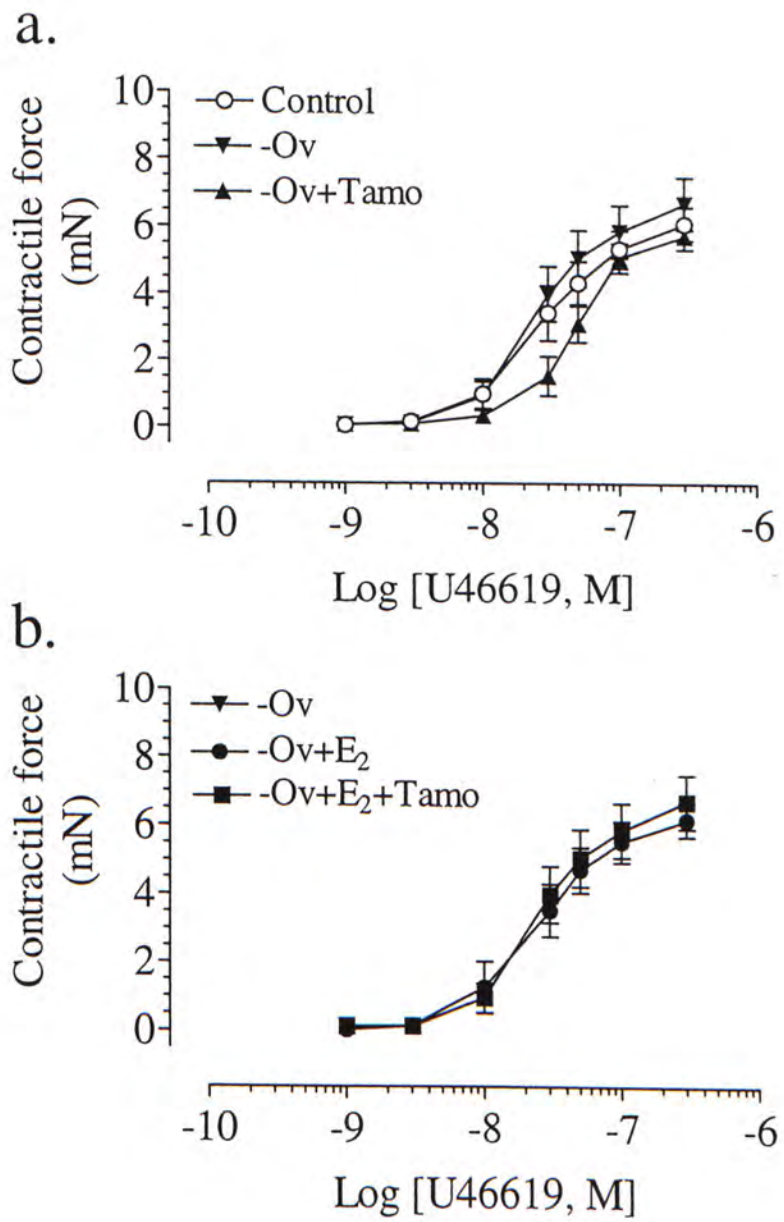

Figure 45

The effect of 17β-estradiol or/and tamoxifen treatment on U46619-induced contraction in endothelium-intact carotid arteries from the ovariectomized rats. (**a**) Concentration-response curves for U46619 in the control group (○, n=6), the ovariectomized group (▼, n=6) and the tamoxifen replaced group (▲, n=4). (**b**) Concentration-response curves for U46619 in the estrogen replaced group (●, n=6), ovariectomized group (▼, n=6); estrogen and tamoxifen replaced group (■, n=6). Data are means ± S.E.M. of *n* experiments.

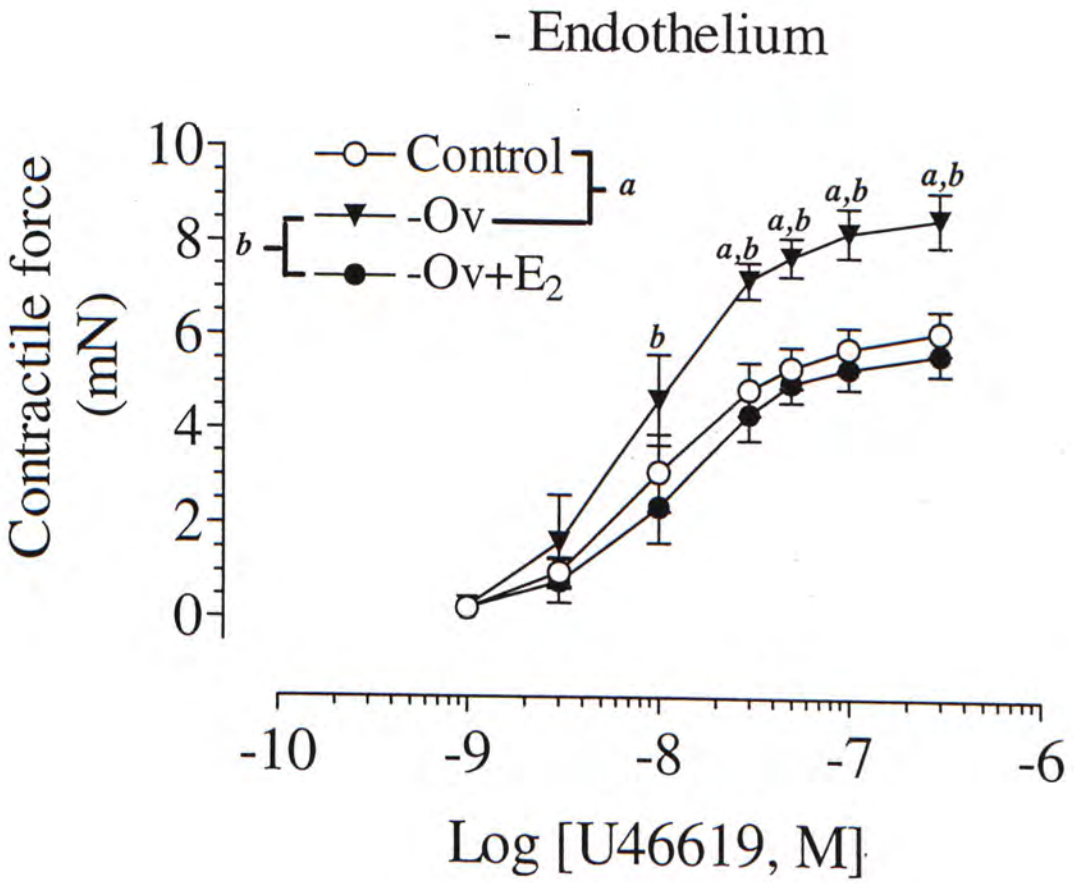

Figure 46

The effect of ovariectomy on U46619-induced contraction in endothelium-denuded rat carotid arteries. Concentration-response curves for U46619 in the control group (○, n=6), the ovariectomized group (▼, n=6), and the estrogen-replaced group (●, n=6). Statistical difference was indicated as ***a*** between the control and ovariectomized groups; ***b*** between the ovariectomized and estrogen-replaced groups ($P < 0.05$). Data are means ± S.E.M. of *n* experiments.

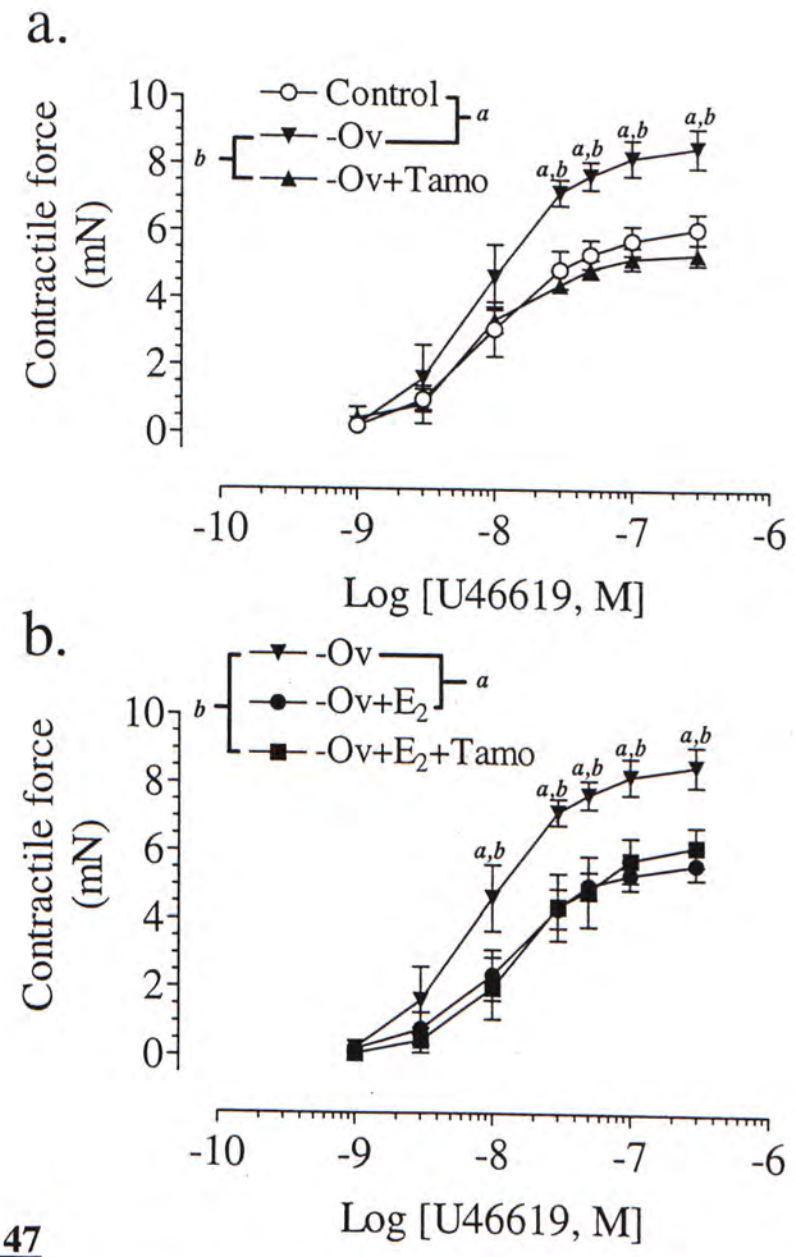

Figure 47

The effect of 17β-estradiol or/and tamoxifen treatment on U46619-induced contraction in endothelium-denuded carotid arteries from the ovariectomized rats. (**a**) Concentration-response curves for U46619 in the control group (○, n=6), ovariectomized group (▼, n=6) and tamoxifen replaced group (▲, n=4). A significant difference ($P < 0.05$) is indicated by ***a*** between the control and ovariectomized groups; ***b*** between ovariectomized and tamoxifen-replaced groups. (**b**) Concentration-response curves for U46619 in the estrogen-replaced group (●, n=6), ovariectomized group (▼, n=6); estrogen and tamoxifen replaced group (■, n=6). Statistical difference was indicated as ***a*** between the ovariectomized and estrogen-replaced groups; ***b*** is between the ovariectomized and co-treatment groups. Data are means ± S.E.M. of *n* experiments.

3.7.1.3. Effect of ovariectomy on high extracellular K^+-induced contraction

In the endothelium-denuded mesenteric arteries, the constrictor response to high extracellular K^+ (5-80 mM) was examined. The vasoconstriction induced high extracellular K^+ was significantly enhanced in the ovariectomized rats (n=5). Traces in Figure 48 shows that 20 mM K^+ produced an increase in vessel tension in an ovariectomized rat (Figure 48b), while this concentration of K^+ produced much smaller contraction in a sham-operated rat (Figure 48a). Figure 49 shows the concentration-response curves for the high K^+ response in endothelium-denuded rings. The curve was significantly shifted upwards in the ovariectomized rats (n=5 in each groups, $P < 0.05$ compared with control values, Figure 49). Estrogen replacement therapy completely abolished the effect of ovariectomy on the vascular response to high K^+ ($P > 0.05$ between the control group, n=5 and estrogen-replaced group, n=5; $P > 0.05$ between the ovariectomized group and estrogen-replaced group, n=5, Figure 49).

Tamoxifen replacement therapy had a similar effect to estrogen. Figure 50a shows that chronic tamoxifen treatment totally reversed the enhancing effect on the high K^+ contraction in the ovariectomized rats ($P < 0.05$ between the control, n=5 and ovariectomized group, n=5). Figure 50b shows that tamoxifen did not additional effect when implanted together with 17β-estradiol pellet. The two concentration-

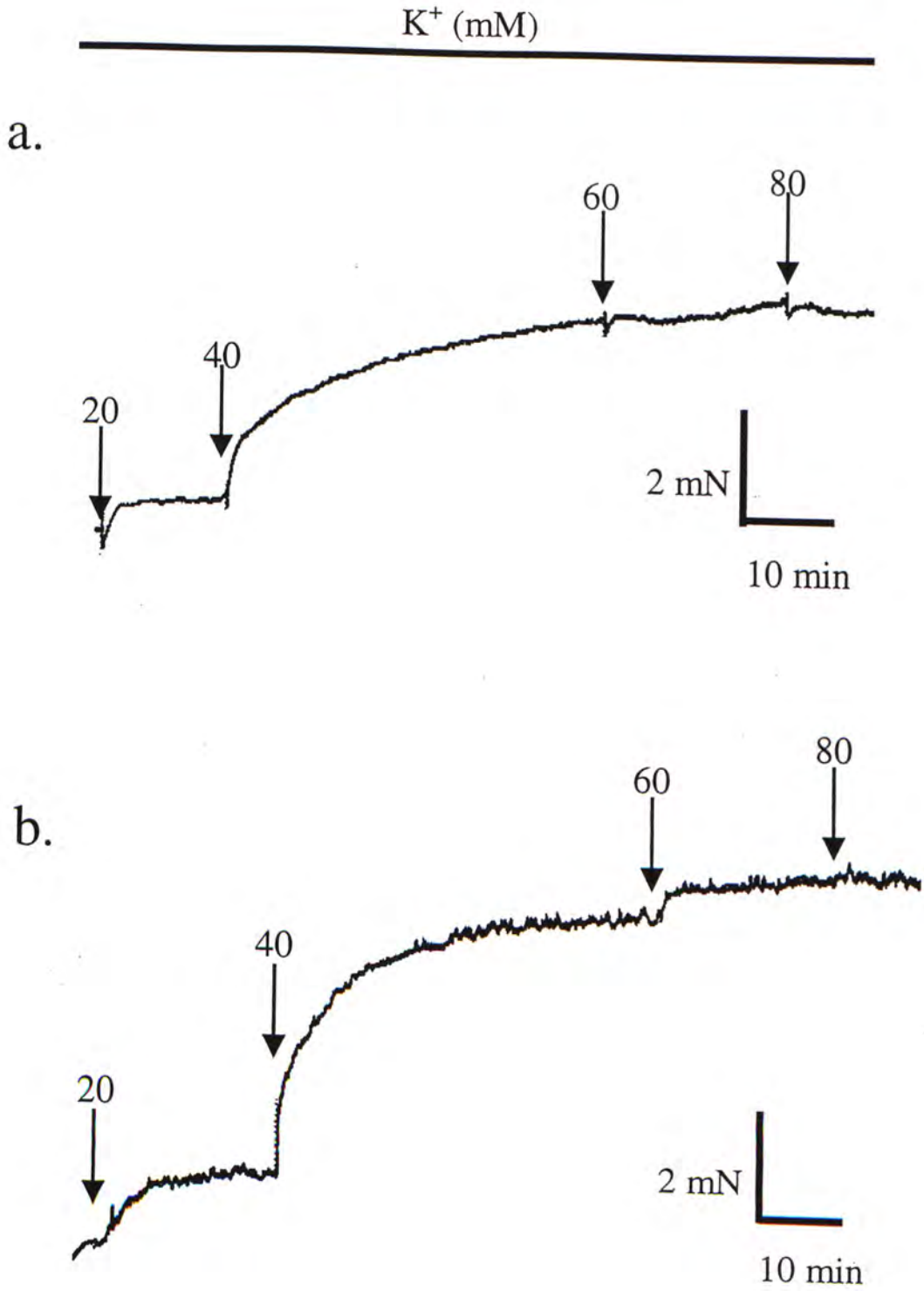

Figure 48

Representative traces showing the contractile response to K^+ in carotid arteries from a control rat (**a**) and an ovariectomized rat (**b**).

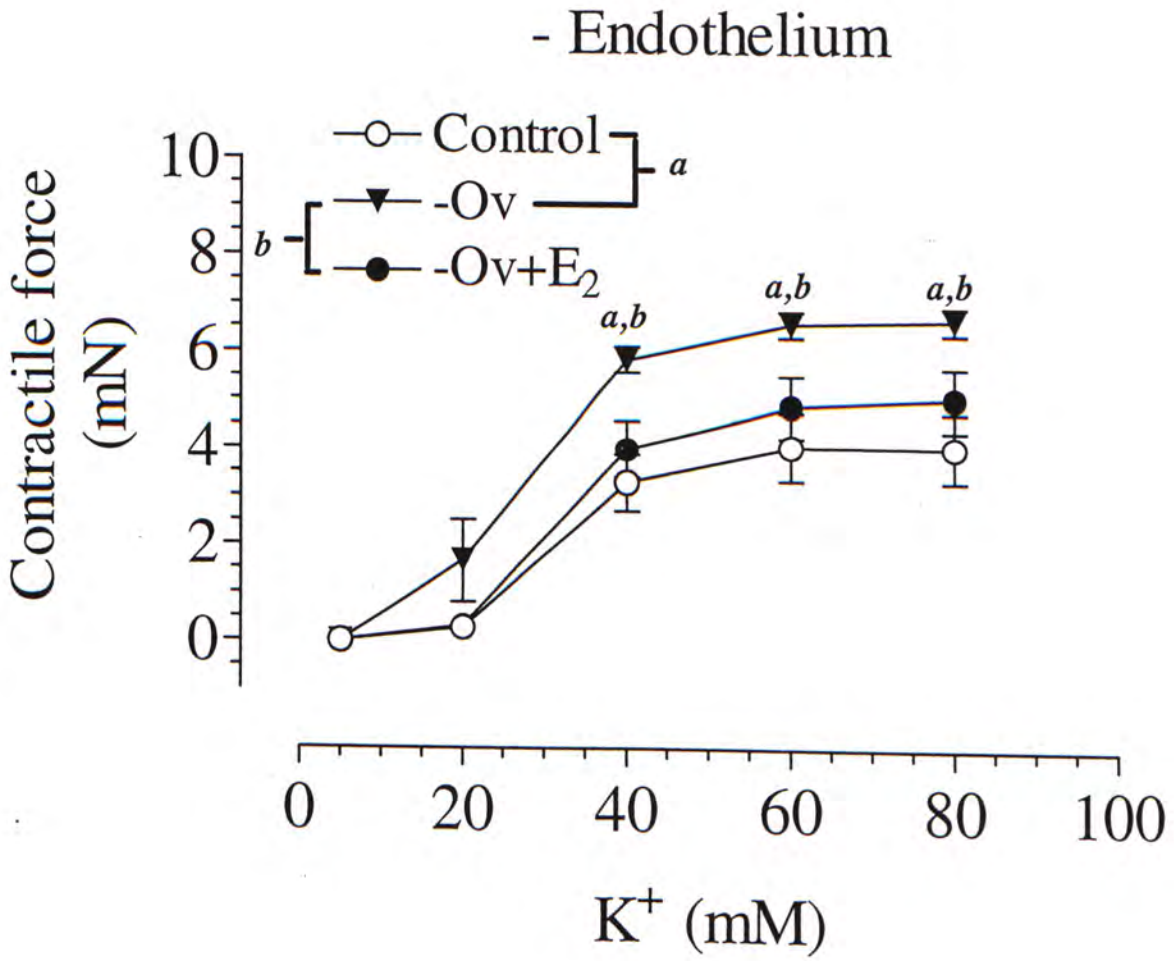

Figure 49

The effect of ovariectomy on the high K^+-induced contraction in rat endothelium-denuded carotid arteries. Concentration-response curves for KCl (○, n=5 for the control group; ▼, n=5 for the ovariectomized group and ●, n=5 for the estrogen-replaced group). A significant difference ($P < 0.05$) is indicated by ***a*** between the control and ovariectomized groups; ***b*** between the ovariectomized and estrogen-replaced groups ($P < 0.05$). Data are means ± S.E.M. of *n* experiments.

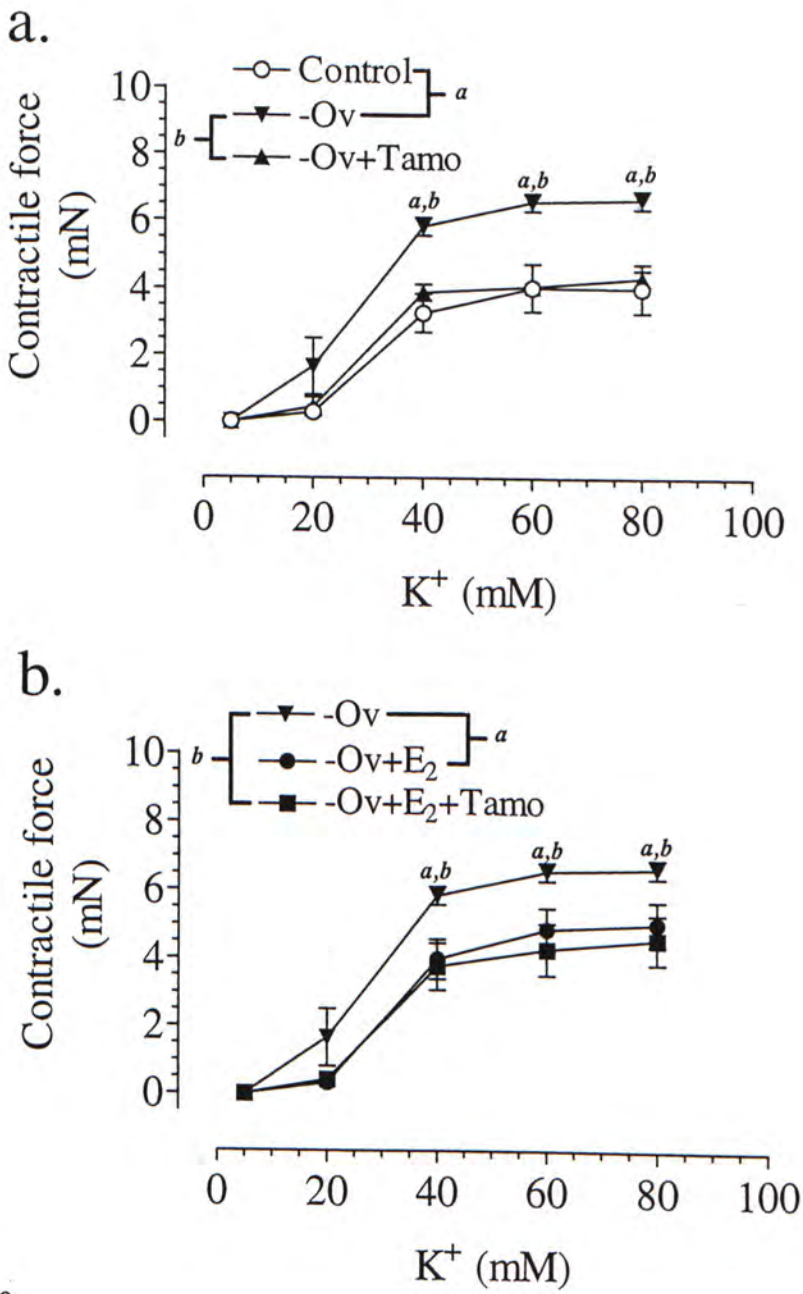

Figure 50

The effect of 17β-estradiol or/and tamoxifen treatment on high K^+-induced contraction in endothelium-denuded carotid arteries from the ovariectomized rats. (**a**) Concentration-response curves for KCl (○, n=5 for the control group; ▼, n=5 for the ovariectomized group; ▲, n=4 for the tamoxifen-replaced group). Significant difference is indicated by ***a*** between the control and ovariectomized groups; ***b*** is between the tamoxifen-replaced and ovariectomized groups. (**b**) Concentration-response curves for KCl (●, n=5 fore the estrogen-replaced group; ▼, n=5 for the ovariectomized group; ■, n=5 for the estrogen and tamoxifen cotreatment group). Statistical difference is indicated by ***a*** between the ovariectomized and estrogen-replaced groups; ***b*** between the ovariectoimized and cotreatment groups ($P < 0.05$). Data are means ± S.E.M. of *n* experiments.

response curves for high K^+ were identical ($P < 0.05$, n=5 in each groups).

3.7.1.4. Effect of ovariectomy on acetylcholine-induced relaxation

In endothelium-intact rat carotid arteries, the effect of ovariectomy on acetylcholine-induced relaxation was also examined. Figure 51 show that estrogen replacement therapy did not modify the endothelium-dependent relaxation induced by acetylcholine. The pD_2 values obtained from the three groups are very similar: 6.82 ± 0.08 for the control group, n=9; 7.12 ± 0.09 for the ovariectomized group, n=4 and 7.17 ± 0.17, n=6 for the estrogen-replaced group ($P > 0.05$).

In addition, chronic treatment with tamoxifen had no effect either. The pD_2 value obtained in Figure 52a: 6.82 ± 0.08 for the control, n=9; 7.12 ± 0.09 for the ovariectomized group, n=4; 6.74 ± 0.09 for the tamoxifen-replaced group, n=6, $P >$ 0.05).

Finally, co-treatment with estrogen and tamoxifen did not influence acetylcholine-mediated relaxant response (pD_2 values: 7.12 ± 0.09 for the ovariectomized group, n=4; 7.17 ± 0.17 for the estrogen-replaced group, n=6; and 7.30 ± 0.11 for co-treatment with estrogen and tamoxifen, n=6) ($P > 0.05$, Figure 52b).

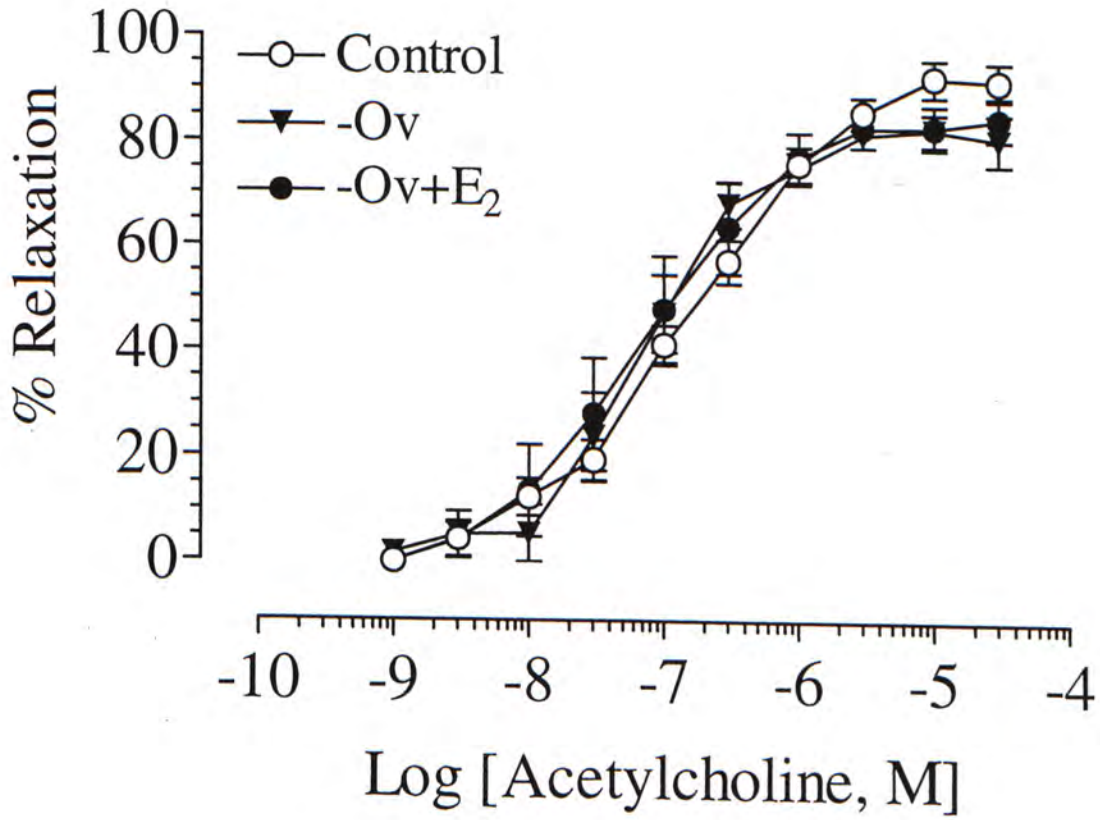

Figure 51

The effect of ovariectomy on acetylcholine-induced relaxation in endothelium-intact rat carotid arteries. Concentration-response curves for acetylcholine in the control (○, n=9), the ovariectomized group (▼, n=4) and the estrogen-replaced group (●, n=6). Data are means ± S.E.M. of *n* experiments.

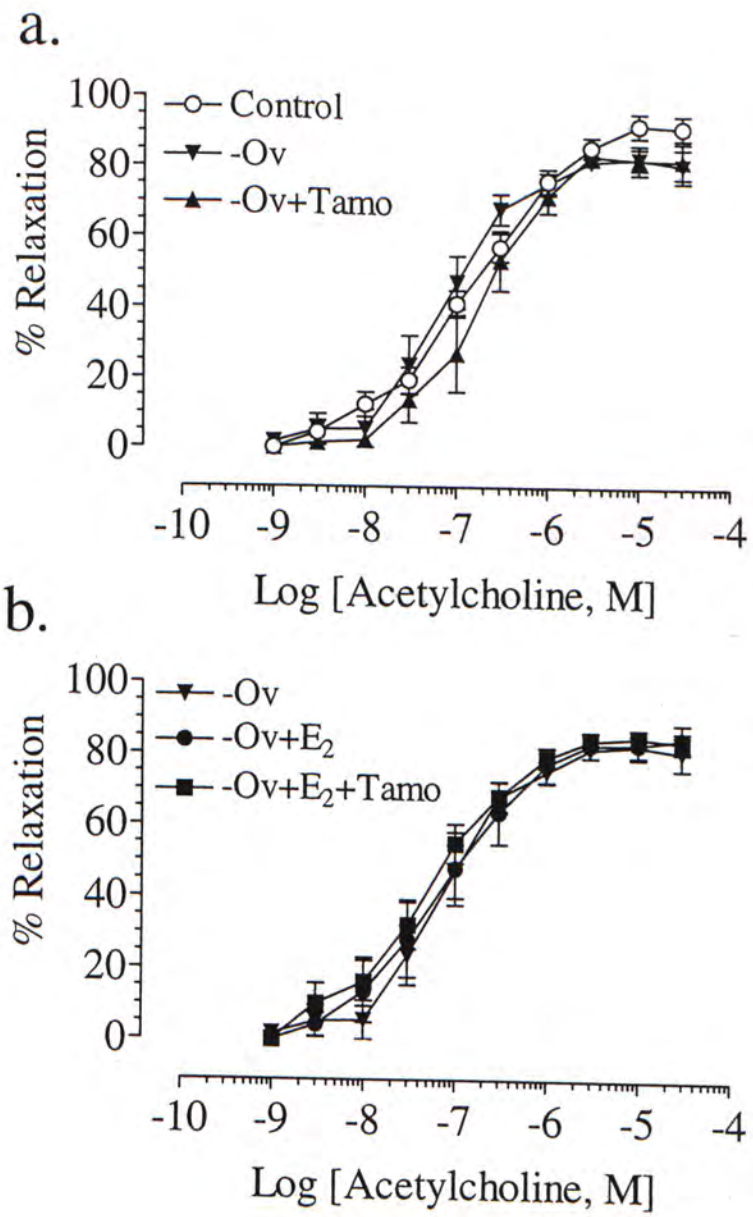

Figure 52

The effect of 17β-estradiol or/and tamoxifen treatment on acetylcholine-induced contractions in endothelium-intact carotid arteries from the ovariectomized rats. **(a)** Concentration-response curves for acetylcholine in the control group (○, n=9), the ovariectomized group (▼, n=4), and the tamoxifen replaced group (▲, n=4). **(b)** Concentration-response curves for acetylcholine in the estrogen-replaced group (●, n=6), the ovariectomized group (▼, n=4); the estrogen and tamoxifen replaced group (■, n=6). Data are means ± S.E.M. of *n* experiments.

Chapter 4 Discussion

Women experience a dramatic increase in the incidence of coronary heart disease following the onset of menopause. Physiological levels of estrogen clearly exert cardioprotective effects since postmenopausal women receiving estrogen replacement therapy are less likely to develop heart disease. The beneficial effects of estrogen include lowering of low-density lipoprotein cholesterol and decreased vascular contractility. However, the precise pathways, and the cellular mechanisms by which female sex steroid hormones influence the activity of various vasculatures are incompletely understood. An increasing amount of evidence suggests that nitric oxide production, and modulation of the vascular responsiveness to endogenous vasoconstrictors or vasodilators may play an important role in the vascular effects of female sex steroid hormones.

I specifically proposed the following hypotheses to be examined. (1) Endothelial nitric oxide may play a differential role in relaxations of arteries of different diameters induced by estrogen and progesterone. (2) β-Adrenoceptor agonists and 17β-estradiol may exert mutual synergistic effects on vessel tone. (3) Sex steroid hormones may inhibit protein kinase C-mediated contraction as a mechanism contributing to their vasodilator effects. (4) Acute exposure to physiological level of 17β-estradiol may modulate β-adrenoceptor-meidated vasorelaxation. (5) Chronic treatment with 17β-estradiol may alter the vascular contractility. To this end, the experiments were carried out on several types of isolated rat arteries, (*e.g.* aorta, mesenteric artery in normal Sprague-Dawley rats and carotid artery from ovariectomized rats), and on the porcine coronary artery to investigate the vascular response to two female sex steroid hormones, 17β-estradiol and progesterone, by measuring changes in vessel tension with Grass force-displacement transducers.

4.1. Role of Endothelium/Nitric Oxide in 17β-Estradiol- and Progesterone-induced Relaxations

The relative protection from cardiovascular disease such as atherosclerosis and hypertension in pre-menopausal women is partly explained by the beneficial effects of estrogen (Stampfer *et al.*, 1991) and it is believed that endothelial nitric oxide is one of the key mediators in the cardioprotective action of female sex steroid hormones. The present results show a different role of the functional endothelium in the relaxant response to 17β-estradiol or progesterone at supra-physiological concentrations in the conduit vessel (aorta) and the smaller-sized vessels (mesenteric artery). Nitric oxide contributes largely to the endothelium-dependent relaxation induced by 17β-estradiol in the isolated aortas or by progesterone in the mesenteric arteries.

In the phenylephrine-preconstricted aortic rings, 17β-estradiol induced both endothelium-independent and –independent relaxation in a concentration-dependent manner. Removal of the endothelium decreased the 17β-estradiol-induced aortic relaxation by approximately 4-fold without an effect on the maximal relaxation (see Figure 9). L-NAME or methylene blue reduced the 17β-estradiol-induced relaxation by a similar extent to that observed in the endothelium-denuded rings. The precursor of nitric oxide synthesis, L-arginine caused a full reversal of the inhibitory effect of L-NAME on 17β-estradiol-induced relaxation (see Figure 10). In rabbit aortas, 17β-estradiol-induced relaxation involved endothelial nitric oxide (Ma *et al.*, 1997). Estrogen was found to enhance NO release in rat aorta (Rahimian *et al.*, 1997). In contrast, endothelium is seemingly not involved in 17β-estradiol-induced relaxation in isolated rat mesenteric arteries since 17β-estradiol relaxed the preconstricted artery rings equi-effectively in both endothelium-intact and –denuded rings. Similarly, N^G-

nitro-L-arginine, another nitric oxide synthase inhibitor did not affect 17β-estradiol-induced relaxation in the pressurized rat small mesenteric arteries (Shaw *et al.*, 2000). The endothelium-independent relaxation to estrogen was also described in the coronary arteries from rats or rabbits (Jiang *et al.*, 1991; Shaw *et al.*, 2000). However, 17β-estradiol-induced flow-dependent relaxation in rat small mesenteric arteries was partly mediated through release of endothelial nitric oxide (Cockell & Poston, 1997). In some studies, the role of nitric oxide in modulating the vascular response to estrogen may be altered by the specificity of the vasoconstrictor used (Zhang & Davidge, 1999) or by different experimental conditions. Nevertheless, our data indicate that in the isolated aortic rings but not mesenteric arteries, 17β-estradiol could act on the endothelium to release nitric oxide as a major mechanism mediating the endothelium-dependent aortic response.

Another female sex hormone progesterone also produced concentration-dependent relaxations in both aortic rings and mesenteric arteries. In contrast to the role of endothelium in the 17β-estradiol-induced relaxation, endothelium/nitric oxide only contributed to progesterone-induced relaxation in the isolated mesenteric arteries but not in the aortas (see Figure 12). Both L-NAME and methylene blue attenuated progesterone-induced relaxation of mesenteric artery rings (see Figure 13). However, progesterone was reported to exert the endothelium-independent relaxation in rabbit and human arteries (Jiang *et al.*, 1992; Omar *et al.*, 1995). It is not known whether this discrepancy is due to vessels prepared from different species. Chronic progesterone was actually found to attenuate estrogen-induced endothelium-dependent relaxation in canine coronary arteries (Miller & Vanhoutte, 1991), however this effect cannot be observed in postmenopausal women (Gerhard *et al.*, 1998). L-Arginine at 1 mM was less effective in antagonizing the effect of L-NAME in

progesterone-induced relaxation of mesenteric arteries while this concentration of L-arginine completely reversed the effect of L-NAME on 17β-estradiol-induced aortic relaxation. It is at present unknown what had caused this discrepancy. It cannot be ruled out that L-NAME may also exert a non-specific inhibitory effect against progesterone-induced relaxation. Non-specific effect of L-NAME reported in endothelium-denuded rabbit coronary arteries was not affected by L-arginine (Buxton *et al.*, 1993).

The present results also show that 17β-estradiol and progesterone displayed different potency in relaxing isolated artery rings. 17β-Estradiol was approximately 3-fold more potent in the aortas than in mesenteric arteries. In contrast, progesterone was about 2.5-fold more potent in mesenteric arteries than in aortas. This difference can be explained by at least one additional mechanism involved in female sex hormone-mediated relaxation, *e.g.* the involvement of endothelium/nitric oxide in the relaxant response of the aortas to 17β-estradiol or in the relaxant response of the mesenteric arteries to progesterone.

Besides, the role of inducible nitric oxide in progesterone-induced relaxation was also examined. In the present study, the effect of AMT HCl at three concentrations (5, 10 and 30 nM) was examined on progesterone-induced relaxation. In isolated rat mesenteric arteries with intact endothelium, AMT, a specific inhibitor of inducible nitric oxide synthase (iNOS), inhibited the progesterone-induced relaxation in a concentration-dependent manner (see Figure 14). Incubation with 30 nM AMT decreased the progesterone-induced relaxation by approximately 4-fold without an effect on the maximal relaxation. Removal of endothelium completely abolished the inhibitory effect of AMT, suggesting that involvement of endothelial iNOS in progesterone-induced relaxation if the concentrations of AMT used are

within the range that still selectively inhibits iNOS. iNOS was previously detected in endothelial cells (Kanno *et al.*, 1994; Aliev *et al.*, 1998). Both progesterone and estrogen were found to inhibit ovariectomy-induced expression of iNOS in female rat aortas (Tamura *et al.*, 2000), however, the location of this effect in the arteries is yet to be determined.

Taken together, I have provided some novel evidence for the different role of endothelium/nitric oxide in relaxations of arteries of different sizes (aortas and mesenteric arteries) induced by 17β-estradiol and progesterone. Endothelium/nitric oxide contributes significantly to 17β-estradiol-induced relaxation of the rat aortic rings or to progesterone-induced relaxation of the rat mesenteric arteries and this contribution to relaxation appears to only relate to lower concentrations of 17β-estradiol and progesterone. It is possible that estrogen and progesterone may stimulate release of endothelial nitric oxide in larger conduit vessels and smaller resistance-sized vessels, respectively, to mediate the beneficial effect in the process of atherosclerosis and hypertension. Besides, endothelial iNOS may also play a role in the relaxant response to progesterone in rat mesenteric artery rings. However, the present studies were carried out on male rats only. Whether female counterparts would respond in a similar manner should be explored in the future.

4.2. Effect of Estrogen Receptor Inhibitor on 17β-Estradiol-induced Relaxation

In the present study, the effect of estrogen receptor inhibitor, ICI 182,780 was examined on 17β-estradiol-induced relaxation. ICI 182,780 did not affect the 17β-estradiol-induced relaxation in endothelium-intact rat mesenteric arteries. Similar

result were also reported in the rat cerebral arteries (Salom *et al.*, 2001) and mesenteric arteries (Shaw *et al.*, 2000). However, ICI 182,780 was found to completely block the 17ß-estradiol-induced increase in intracellular [Ca^{2+}] in cultured endothelial cell, but tamoxifen, an estrogen agonist/antagonist was devoid of effect (Rubio-Gayosso *et al.*, 2000). Besides, prior administration of ICI 182,780 blocked the estrogen-induced changes in baroreflex sensitivity and autonomic tone (Saleh *et al.*, 2000). Other investigators also reported that ICI 182,780 could completely inhibit the estrogen-stimulated NO release (Yang *et al.*, 2000; Kim *et al.*, 1999). The discrepancy may be due to the difference in the receptor targets for ICI 182,780, estrogen receptor α and β forms in blood vessels (Pace *et al.*, 1997). These data suggest that it is possible that the vascular response of different vessels to 17β-estradiol may be mediated through different sub-types of estrogen receptors.

4.3. Interaction between Progesterone and 17β-Estradiol

In the present study, supra-physiological concentrations of progesterone (0.3 and 1 μM) were found to potentiate the 17β-estradiol-induced relaxation in endothelium-intact mesenteric artery rings (4-fold reduction in IC_{50} value, see Figure 16) without affecting the maximal relaxation. However, this enhancing effect was abolished following removal of the endothelium, suggesting that progesterone increased the relaxant effect of 17β-estradiol via an endothelium-dependent mechanism. Due to the time constraint, no attempt was made to identify what endothelium-derived vasoactive factors may be involved in the potentiating effect of progesterone or to examine whether 17β-estradiol should have the same effect as progesterone in rat mesenteric arteries. Paradoxically, other investigators reported that progesterone opposed the vascular effect of 17β-estradiol (Miyagawa *et al.*, 1997).

Also, short-term exposure to 1 nM progesterone reduced the enhancing effects of 17β-estradiol on the bradykinin and A23187-induced vasorelaxation (Teoh & Man, 1999). This discrepancy may result from the different incubation time with progesterone. In my study, the arterial rings were allowed to incubate for 2.5 hours with progesterone; however, the incubation time of Teoh and Man's study was not more than 30 minutes. Moreover, the concentration of progesterone used in this study was much higher than 1 nM, a concentration used by Teoh and Man. Interestingly, the present results show that the presence of endothelium is required for the enhancing effect of progesterone. As discussed in Section 4.1., progesterone induced both endothelium-dependent and –independent relaxation, while 17β-estradiol produced only endothelium-unrelated relaxation in the isolated rat mesenteric arteries. It is therefore possible that progesterone at 0.3 μM may have released some NO in the endothelium, which then synergistically interact with 17β-estradiol. If this proves to be a case, low concentration of NO donors should also enhance estrogen-induced relaxation in the endothelium-denuded rings. This likelihood will be tested in the future study.

4.4. Effects of Female Sex Steroid Hormones on Protein Kinase C-mediated Contraction

Estrogen has been widely reported to relax blood vessels induced by various constrictors such as noradrenaline, endothelin, prostaglandins and angiotensin II. Vasoconstriction induced by these agents are partly mediated through activation of protein kinase C (PKC) subsequent to production of diaceylglycerol, an endogenous stimulator of PKC in vascular SMC. It is therefore possible that estrogen may

interfere with PKC-dependent mechanism leading to vessel contraction. The present experiments were designed to test this possibility. PDA, an active phorbol ester which activates PKC, induced a slow-developing tension in endothelium-denuded rings bathed in Ca^{2+}-free Krebs solution, indicating that PDA-induced contraction is not associated with Ca^{2+} influx; and PKC activation can interact with contractile filaments at the resting $[Ca^{2+}]_i$ in vascular SMC (Huang, 1996). In Ca^{2+}-containing Krebs solution, PDA induced similar level of vessel tone but the onset of response was faster, probably due to additional mechanisms involved. It was reported that Ca^{2+} entry in vascular smooth muscle was activated during PKC activation by phorbol ester (Gleason & Flaim, 1986).

17β-Estradiol and progesterone produced concentration-dependent relaxation in rings contracted by PDA (see Figures 17 & 18) with the former being more effective. In the absence of extracellular Ca^{2+} ions, the relaxing effect of both hormones was significantly enhanced at most concentrations tested.

In non-vascular preparations, 17β-estradiol stimulated a Ca^{2+} influx in rat colonic crypts which was blocked in the presence of zero extracellular Ca^{2+} (Doolan *et al.*, 2000). It appears to a marked difference in the effect of estrogen on Ca^{2+} influx in vascular and non-vascular cells.

4.5. Effects of β-Adrenoceptor Agonists on 17β-Estradiol-induced Relaxations

The results of this investigation provide evidence for the enhanced relaxant response to female sex steroid hormone, 17β-estradiol in the isolated rat mesenteric artery rings pretreated with low concentrations of isoproterenol, a non-selective β-

adrenoceptor agonist. It is known that isoproterenol induces both endothelial nitric oxide-dependent and -independent relaxation in the coronary arteries of other species (Parent *et al.*, 1993; Lu *et al.*, 1995).

It appears that this enhancing effect is dependent on the presence of the functional endothelium since removal of the endothelium or inhibition of nitric oxide synthase by L-NAME abolished the effect of isoproterenol (see Figures 22 & 23). Moreover, pretreatment with the membrane permeable inhibitor of cyclic GMP-dependent protein kinase, Rp-cGMPS triethylamine also significantly attenuated the isoproterenol-induced potentiation. On the other hand, the endothelium was not involved in 17β-estradiol-induced relaxant response in the same preparations (Chan *et al.*, 2001). The role of endothelial nitric oxide in isoproterenol-induced relaxation was demonstrated in rat mesenteric arteries (Graves & Poston, 1993; Huang *et al.*, 1998) and rat aorta (Gray & Marshall, 1992; Delpy *et al.*, 1996). L-NAME or methylene blue (an inhibitor of guanylyl cyclase) significantly reduced the relaxant response to β-adrenoceptor agonists or forskolin, an adenylyl cyclase inhibitor in these blood vessels. Forskolin also induced endothelium-dependent aortic relaxation (Gray & Marshall, 1992; Toyoshima *et al.*, 1998), indicating a role of endothelial nitric oxide in relaxation initiated by cyclic AMP-elevating dilators.

My data demonstrate that isoproterenol-induced potentiation of 17β-estradiol-induced relaxation was abolished by Rp-cAMPS triethylamine, a membrane permeable protein kinase A inhibitor (see Figure 24). Pretreatment with low concentration of cyclic AMP-elevating agent, forskolin, also amplified the relaxant effect of 17β-estradiol in endothelium-intact rings (see Figure 25). These results suggest that the effect of isoproterenol may be mediated through cyclic AMP-dependent mechanism in the endothelium. Both isoproterenol and forskolin

stimulated production of cyclic AMP and cyclic GMP in endothelium-intact rat aortic rings (Gray & Marshall, 1992).

Crosstalk may occur between cyclic GMP- and cyclic AMP-dependent cellular pathways. Low concentrations of nitrovasodilators exerted marked synergistic interaction with isoproterenol, enhancing isoproterenol-induced aortic relaxation (Delpy *et al.*, 1996; Maurice *et al.*, 1991). Reciprocally, isoproterenol and forskolin were reported to potentiate sodium nitroprusside-induced increase in cyclic GMP levels in rat brain capillary endothelial cells (Vigne *et al.*, 1994). In cultured porcine aortic endothelial cells, isoproterenol and forskolin amplified bradykinin- and ATP-induced formation and secretion of endothelium-derived relaxing factor; this effect corresponded to the enhanced cyclic AMP-elevating agent-induced increase in intracellular free Ca^{2+} concentration (Graier *et al.*, 1992). These studies suggest the presence of extensive crosstalk among cyclic AMP, cyclic GMP and probably also Ca^{2+}-signaling mechanisms to jointly regulate endothelial functions. In isolated canine coronary microvessels, isoproterenol, forskolin and bromo-cyclic AMP increased nitric oxide production. Both L-NAME and Rp-cAMPS markedly inhibited the nitric oxide release induced by these cyclic AMP-elevating agents. Forskolin also enhanced nitric oxide production stimulated by bradykinin in coronary blood vessels (Zhang & Hintze, 2001). These new findings indicate a cyclic AMP-dependent endothelial nitric oxide-forming pathway in coronary blood vessels, which may play an important role in the regulation of vessel tone. This may explain the endothelium-dependent component of the relaxant responses to other endogenously occurring cyclic AMP-elevating transmitters and neuropeptides such as adrenomedullin and calcitonin gene-related peptides in some mammalian blood vessels (Gray & Marshall, 1992; Hayakawa *et al.*, 1999).

In rat aortic rings, the relaxant response to isoproterenol or dibutyryl AMP was inhibited by L-NAME. Isoproterenol-induced relaxation was associated with increases in tissue cyclic AMP and cyclic GMP contents. L-NAME reduced isoproterenol-induced increase in cyclic GMP but not cyclic AMP levels (Toyoshima *et al.*, 1998). It seems that the relaxant response of rat aorta to cAMP-mediated vasodilators is mediated at least in part through nitric oxide production in endothelium and subsequent increase in cyclic GMP in vascular smooth-muscle cells. Our results indicate that isoproterenol-induced increase in endothelial cyclic AMP level may enhance the basal nitric oxide production and release, which subsequently interacts synergistically with 17β-estradiol to produce greater relaxation. Therefore, the amplifying effect of isoproterenol could be mimicked by forskolin and abolished by the inhibitors of either protein kinase A or nitric oxide synthase. To certain degree current data support a previous study in which histamine-induced nitric oxide release was significantly reduced by Rp-cAMPS, suggesting that in porcine aortic endothelial cells, nitric oxide-mediated vasodilation might be caused by production of cyclic AMP initiated through the histamine H-receptors (Kishi *et al.*, 1998).

Both β_1 and β_2-adrenoceptor agonists produced endothelium-dependent and –independent relaxation in rat mesenteric arteries (Huang & Kwok, 1997; Huang *et al.*, 1998) and in rat aorta (Toyoshima *et al.*, 1998). Stimulation of cyclic AMP formation in the endothelium by isoproterenol, leading to direct or indirect release of nitric oxide, was reported by Gray and Marshall (1992); however, the subtype of the β-adrenoceptors involved was not further determined. The present results show that isoproterenol-induced enhancement was partially antagonized by propranolol (see Figure 27), a non-selective β-adrenoceptor antagonist but almost completely inhibited by ICI 118,551 (see Figure 29), a selective β_2-adrenoceptor antagonist (O'Donnell &

Wanstall, 1981). In contrast, atenolol, a selective β_1-adrenoceptor antagonist (O'Donnell & Wanstall, 1981) did not influence the effect of isoproterenol. These results clearly point to a primary role for β_2-adrenoceptors in the enhanced relaxant response to 17β-estradiol in isoproterenol-pretreated rings. My results support a recent *in vivo* study with perfused rabbit femoral artery (Xu *et al.*, 2000). Isoproterenol decreased prefusion pressure via stimulation of β_2-adrenoceptor subtype. Injection of L-NAME abolished the pressure response to isoproterenol and significantly suppressed the pressure response to forskolin and dibutyryl cyclic AMP (Xu *et al.*, 2000). These results together with my findings again indicate that β_2-adrenergic stimulation and cyclic AMP elevation activate a nitric oxide-producing pathway in mammalian arteries *in vitro* and *in vivo*.

In summary, I have provided novel mechanistic evidence for the isoproterenol potentiation of 17β-estradiol-induced relaxant response in rat mesenteric arteries. Isoproterenol may stimulate endothelial β_2-adrenoceptors to elevate basal nitric oxide release via a cyclic AMP-dependent mechanism on the endothelium. This increased level of nitric oxide then interacts synergistically with cellular mechanisms initiated by 17β-estradiol. There may exist a crosstalk linkage between cyclic AMP and cyclic GMP in the endothelium, which could play an important role in the control of vessel tone by more than two endogenous dilators utilizing cyclic nucleotide-mediated intracellular pathways. This has been the first attempt to investigate the synergistic interaction between β-adrenoceptor agonists and estrogen in the arterial rings *in vitro*. It is unclear whether sympathetic neurotransmistters such as adrenaline and noradrenaline at physiological levels would enhance the vasodilator effect induced by 17β-estradiol at circulating concentrations *in vivo*.

4.6. Effects of 17β-Estradiol on β-Adrenoceptor Agonists-induced Relaxations in Porcine Coronary Artery

Considerable evidence suggests that β-adrenoceptors are subject to regulation by sex steroid hormones. For example, intrauterine application of 17β-estradiol significantly up-regulated expression of guinea-pig myometrial β-adrenoceptors (Hatijis *et al.*, 1989). Progesterone increased the number of β-adrenoceptors in the brain of adult, ovariectomized rats (Maggi *et al.*, 1985). Estrogen and progesterone exerted synergistic effect in increasing the β-adrenoceptor density in rat heart (Klangkalya and Chan, 1988). The similar influence of estrogen or progesterone on the number of β-adrenoceptors was also observed in rabbit lung tissue (Moawad *et al.*, 1982) and rat liver (Yagami *et al.*, 1994). Ferrer *et al.* (1996) provided evidence that estrogen replacement enhanced β-adrenoceptor-mediated relaxation in rat mesenteric arteries. It was suggested that estrogen replacement potentiated vascular responses induced by β-adrenoceptor activation by an endothelium-independent mechanism (Ferrer *et al.*, 1996), even though isoproterenol was reported to induce both endothelium-dependent and –independent relaxation in the same preparations (Huang & Kwok, 1997). The same research group also described that estrogen replacement enhanced vasoconstriction induced by smooth-muscle α_2-adrenoceptor activation, although this effect was obscured in intact vessels due to an overriding influence of endothelial dilator substances, primarily NO. In mesenteric arteries from ovariectomized rats, smooth muscle was less sensitive to α_2–adrenoceptor agonist stimulation, however, the release of a vasoconstrictor prostanoid from the endothelium was predominant, and induced significant vasoconstriction. (Ferrer & Osol, 1998). β-Adrenoceptor is present on both smooth-muscle (Asano *et al.*, 1991)

and endothelial cells (Molenaar *et al.*, 1988). However, no experiment has examined the effect of acute exposure to physiological concentrations of estrogen on ß-adrenoceptor –mediated vasorelaxation in mammalian arteries.

The major observation in this series of experiments is that the relaxant response to β_2-adrenoceptor activation with fenoterol was significantly enhanced by short-term incubation (1 hr) with 0.3 nM 17β-estradiol in the isolated porcine coronary circumflex arteries (see Figure 32 & 33b). The concentration of 0.3 nM falls into the reported circulating level of estrogen ranging between 0.1 and 1 nM in the body (Abraham *et al.*, 1972; Rosano *et al.*, 1993; Gilligan *et al.*, 1994). In contrast, 20-min exposure to 17β-estradiol (0.1-1 nM) was without effect on β_2-adrenoceptor-induced response. Low concentrations of estrogen also appeared to slightly enhance isoproterenol-induced relaxation following 1-hr incubation. Fenoterol is a selective β_2-adrenoceptor agonist, which induced both endothelium-dependent and –independent vasorelaxation (Huang & Kwok, 1997), while isoproterenol is a non-selective β- (β_1 and β_2) adrenoceptor agonist. This difference may partly explain a greater potentiating effect of estrogen on relaxation induced by fenoterol than isoproterenol if β_2-adrenoceptor-mediated response is predominant in the porcine coronary arteries. It is interesting to note that 1 nM 17β-estradiol (1-hr exposure) did not modulate fenoterol-mediated coronary relaxation. It is not known whether estrogen may have different modulatory effect on β-adrenoceptor-mediated cellular response depending upon the concentration used. Alternatively, estrogen exhibits differential sensitivity in different steps in intracellular second messenger pathways activated by β_2-adrenoceptor stimulation in intact blood vessels. Further experiments are apparently required to examine whether higher concentrations of estrogen (> 10 nM) could have inhibitory effect on β_2-adrenoceptor-mediated vasorelaxation. Even

though β_1-adrenoceptor agonist such as dobutamine also caused vasorelaxant effect (Huang *et al.*, 1998), short-term exposure to estrogen (0.1-1 nM, either 20-min or 1-hr) did not enhance dobutamine-induced relaxation in porcine coronary arteries. Instead, 1-hr incubation with 17β-estradiol reduced relaxation induced by dobutamine (see Figure 37). If 0.3 nM estrogen enhanced β_2-adrenoceptor-mediated relaxation on one hand, and reduced β_1-adrenoceptor-mediated relaxation on the other hand, it would be not surprising to observe a marginal effect of estrogen on relaxation induced by isoproterenol (see Figure 31), an agonist that activates both β_1 and β_2-adrenoceptors in blood vessels.

My results were inconsistent with a previous study with non-vascular smooth muscle. 17β-Estradiol was found to increase the isoproterenol-induced relaxation but not the fenoterol-induced relaxation in the porcine bronchus (Foster *et al.*, 1983). These discrepancy likely results from the difference in experimental tissues used in these two studies. Other studies with non-vascular tissues demonstrated that lymphocyte β_2-adrenceptor function in normal females was under the cyclical influence of ovarian sex steroids, with greater β_2-adrenceptor density and cAMP response to isoproterenol during the luteal phase than the follicular phase. These effects were associated with elevated post-ovulatory levels of progesterone and estrogen (Wheeldon *et al.*, 1994).

The present results also show that the enhancing effect of 0.3 nM estrogen on fenoterol-induced relaxation can be reversed by 10 μM tamoxifen, a reported mixed estrogen agonist/antagonist. Tamoxifen produced a small but insignificant inhibition of fenoterol-induced response and no explanation could be provided at present. This indicates that tamoxifen may not act in the same manner as estrogen, otherwise, both compounds should have similar effect on the relaxant response to β_2-adrenoceptor

agonist. It remains to be verified whether the antagonistic effect of tamoxifen is mediated through binding to the sites as the same as for estrogen in vascular tissue. Tamoxifen has been recently demonstrated to relax rabbit coronary arteries via an endothelial NO- and estrogen receptor-dependent mechanism (Figtree *et al.*, 2000). Tamoxifen was also found to inhibit voltage-operated Ca^{2+} channels in vascular smooth muscle cells and this mechanism may contribute to its inhibitory effect on vasoconstriction (Song *et al.*, 1996).

Increased levels of cAMP are thought to mediate β-adrenoceptor-induced vasorelaxation (Phornchirasilp & Lockwood, 1977). Estrogen (supra-physiological level)-mediated endothelium-independent relaxation may be also partly caused by elevated cAMP contents in blood vessels (Mugge *et al.*, 1993). However, the physiological concentration of 17β-estradiol was found to have no effect on cAMP contents in porcine coronary smooth muscle cells (Christ *et al.*, 1999). In order to examine the possibility that the enhancing effect of estrogen on fenoterol-induced relaxation is associated with intracellular cAMP that could be raised by β-adrenoceptor stimulation, the effect of acute 17β-estradiol was tested on relaxation induced by IBMX, a phosphodiesterase inhibitor. Short-term exposure to 0.1-1 nM estrogen did not have a significant effect despite 20-min incubation with estrogen seems to have a slight enhancing effect. However, it was reported that 17β-estradiol enhanced the relaxant potency of another phosphodiesterase inhibitor, theophylline (Foster *et al.*, 1983). More experiments are certainly needed to examine this hypothesis by measuring cAMP in endothelium-intact porcine coronary arteries under the same treatment as that in contraction experiments.

4.7. Effect of Ovariectomy on the Vascular Reactivity

A previous study showed that the maximal pressor response to phenylephrine is greatly attenuated in aortas from female rats as compared with that from male rats (Stallone, 1993). In addition, 17β-estradiol was shown to significantly decrease the contractile response to phenylephrine in aortic rings of male rats (Thomas *et al.*, 1995). Long-term 17β-estradiol treatment reduced the contraction of rat aorta to phenylephrine (Andersen *et al.*, 1999). These data indicate that long-term inhibitory influence of estrogen on adrenergically mediated vasoconstriction. In order to investigate whether physiological levels of estrogen in rat arteries could modify the constrictor response, I also examined the impact of ovariectomy and 17β-estradiol replacement therapy on the vascular reactivity in normal control rats and ovariectomized female rats. The common carotid artery was used which may resemble the properties of cerebral arteries supplied by the internal carotid artery.

The present study shows that in endothelium-intact carotid arteries, ovariectomy did not significantly affect the concentration-dependent contractile response to phenylephrine even though phenylephrine-induced tension tended to increase. 17β-Estradiol replacement treatment brought back this small enhancing effect. In contrast, the phenylephrine-induced contraction was markedly increased in ovariectomized rats and this enhancement can be partially inhibited by 17β-estradiol replacement (see Figure 42). It is unknown whether partial inhibition by chronic estrogen is due to more dominating effect of phenylephrine on the endothelium following ovariectomy. Meyer *et al.* reported that endothelium-intact mesenteric arteries from estrogen-replaced rats were significantly less sensitive to phenylephrine (Meyer *et al.*, 1997; Zhang & Davidge, 1999). Some explanations were given that α_1-adrenergic receptor expression may be decreased in the mesenteric arteries from 17β-

estradiol-replaced rats as compared with those from the ovariectomized animals (Zhang & Davidge, 1999). Furthermore, the maximal binding of α_1-adrenoceptors in cardiac ventricles was found to be greater in male than in female Dahl rats (Hayahi *et al.*, 1995). However, this potential mechanism can not explain my results with phenylephrine since phenylephrine produced greater contractile response in endothelium-denuded rings than endothelium-intact rings in three groups of rats. One possibility is that α_1-adrenoceptor-mediated endothelium-dependent relaxing effect may remain intact or even enhanced in the ovariectomized carotid arteries. This effect would apparently counteract with increased contractility in SMC induced by phenylephrine. An endothelium-dependent response was observed in vascular response to both α_1 and α_2-adrenoceptor stimulation (Tabernero *et al.*, 1999; Nishina *et al.*, 1999).

These data are consistent with a report by Binko and Majewski who showed that 24-hour incubation with 17β-estradiol of isolated endothelium-denuded aortic rings from ovariectomized rats attenuated the phenylephrine-induced constriction (Binko & Majewski, 1998). In addition, this attenuation was also observed even the treatment was shortened to 6 hours (Binko *et al.*, 1998). Paradoxically, other researchers reported no difference in phenylephrine-induced contraction in endothelium-denuded mesenteric arteries (Meyer *et al.*, 1997) or rat aorta (Andersen *et al.*, 1999) between the ovariectomized and estrogen-replaced groups. The exact cause between this difference is unclear, but it may related to different arteries used by different research groups or different methods used for chronic estrogen treatment (injection of low concentrations of estrogen or estrogen pellet implantation which allows stable daily release of estrogen).

Similar to the results with phenylephrine, U46619-induced contraction remains the same in rat carotid arteries prepared from the control, ovariectomized and estrogen-replaced groups (see Figure 44). Similar data were also obtained in rat mesenteric arteries in which the vascular response to U46619 did not differ between the estrogen-pretreated and ovariectomized rats (Zhang & Davidge, 1999). In addition, Miller and Vanhoutte described failure of estrogen to modify the vascular contractility to U46619 in the rabbit aortas (Miller & Vanhoutte, 1990). In contrast, one study did show that estrogen potentiated the responsiveness of mesenteric arteries to U46619 in male rats (Vargas *et al.*, 1995).

On the other hand, when the endothelium was mechanically denuded, the contractile response to U46619 was increased in the ovariectomized rats compared with the control rats (see Figure 46). This enhancement was completely reversed by estrogen replacement therapy. Similar results were reported in the endothelium-denuded coronary and renal arteries in which U46619 produced higher vessel tension in male than female dogs (Karanian & Ramwell, 1996). In addition, estrogen treatment in male dogs decreased the pressor response of U46619 (Karanian & Ramwell, 1996). These findings again indicate that function of the endothelium may be normal or even enhanced, as a result, the increased contraction of SMC to U46619 or phenylephrine may have been masked in the endothelium-intact rings.

In addition to phenylephrine and U46619, I also examined the effect of high K^+ on vessel tone to rule out the possibility that changes in vascular contractility is associated with vasoconstrictors whose action is mediated by intracellular second messengers. In endothelium-denuded carotid arteries, both maximal arterial contraction and sensitivity to K^+ were increased in the ovariectomized rat (see Figure 49). The effect of ovariectomy on high K^+ response was totally inhibited by estrogen

replacement. Andersen *et al.* reported that the maximal aortic contraction to K^+ was attenuated in the estrogen-replaced rats (Andersen *et al.*, 1999). Moreover, high K^+-induced contraction was smaller in aortas from the pregnant rats (Honda *et al.*, 1996), indicating the chronic influence of estrogen in the body on Ca^{2+} influx through plasma membrane Ca^{2+} channels.

Another novel finding of the present study is that tamoxifen replacement therapy displayed the same effect as estrogen replacement therapy on vascular contractility. In the endothelium-denuded rings, the magnitude of the contractile response to phenylephrine, U46619, or high K^+ is similar in three groups of rats, *e.g.* the control, estrogen-replaced and tamoxifen-replaced groups (see Figures 43, 47 & 50). However, contradictory results were presented that tamoxifen increased the pressor response to U46619 in the endothelium-denuded arteries of female dogs (Karanian & Ramwell, 1996). Besides, chronic co-treatment with estrogen and tamoxifen did not have additive effect on contraction induced by U46619 or high K^+. But co-treatment seems to have a greater effect on phenylephrine-induced contraction than single treatment (see Figure 43). These new results clearly indicate that chronic tamoxifen may not act as an anti-estrogen, rather, it behaves like an estrogenic agent.

The present study also examined influence of ovariectomy on the endothelium-dependent relaxation in the rat carotid arteries. Acetylcholine produced concentration-dependent relaxation, which was sensitive to inhibition of inhibitors of NOS. This relaxation was unaltered in the ovariectomized rats (see Figure 51). There was no difference in acetylcholine-induced relaxation in the control, ovariectomized, estrogen-replaced, tamoxifen-replaced groups and co-treatment with estrogen and tamoxifen (see Figure 52). These data agreed with the results obtained by White *et al.* who showed that the concentration-response curve to acetylcholine in left carotid

arteries was similar in male, vehicle-treated female and 17β-estradiol-supplemented female rats (White *et al.*, 1997). In addition, other investigators demonstrated that chronic treatment with estrogen did not influence the acetylcholine-induced relaxation of rat aorta (Meyer *et al.*, 1997). However, conflicting data have also been provided. Prolonged treatment with estrogen potentiated the acetylcholine-induced relaxation in rat aorta (Cheng *et al.*, 1994; Binko & Majewski, 1998; Andersen *et al.*, 1999), rabbit femoral arteries (Gisclard *et al.*, 1988), canine coronary arteries (Miller & Vanhoutte, 1991), and guinea pig heart (Thompson *et al.*, 2000). In addition, the relaxant response to acetylcholine was greater in the aortas from the pregnant rats than non-pregnant rat (Honda *et al.*, 1998). Cheng *et al.* explained that the enhanced acetylcholine-induced responses may be due to depressed acetylcholinesterase activity (Cheng *et al.*, 1994). Whatever mechanisms are involved in improved acetylcholine-mediated relaxation by estrogen in a number of arteries, my results do not suggest the modulatory effect of chronic estrogen on acetylcholine-induced endothelium-dependent relaxation under my experimental condition. However, this piece of data, together with results with vasoconstrictors, provides additional information suggesting the endothelium may function normally after ovariectomy. It would be better to have examined the endothelium-dependent relaxation in different arterial rings to see whether there is regional difference in vasculature in response to acetylcholine or other endothelium/nitric oxide dilators. Unfortunately, I was unable to perform all these experiments due to the time constraint.

4.8. Conclusions

In conclusion, my results have provided some novel findings concerning the arterial effects of female sex steroid hormones. Firstly, the endothelial nitric oxide

plays different role in the relaxant response to 17β-estradiol and progesterone in the conduit vessel (aorta) and the smaller-sized vessels (mesenteric artery). Endothelial nitric oxide contributes largely to the endothelium-dependent relaxation induced by 17β-estradiol in the isolated aortas or by progesterone in the mesenteric arteries. Secondly, 17β-estradiol-induced relaxation in the mesenteric arteries was enhanced by isoproterenol, primarily through activation of β_2-adrenoceptors. Pretreatment with inhibitors of nitric oxide activity, such as L-NAME or Rp-8-cGMPS eliminated or markedly inhibited the effect of isoproterenol, suggesting that isoproterenol may act on the endothelial cells to release nitric oxide and cyclic GMP-dependent pathway was involved in the effect of isoproterenol. Cyclic AMP-dependent mechanism in endothelium was also involved in the effect of isoproterenol. These new findings suggest that β_2-adrenergic agonist and 17β-estradiol can synergies each other via several intracellular second messengers primarily in the endothelium. Thirdly, both 17β-estradiol and progesterone concentration-dependently reduced the contractile response to the activator of protein kinase C, indicating an additional cellular mechanism by which steroid hormones reduce vessel tone evoked by PKC-dependent constrictors such as phenylephrine, endothelin and angiotensin II. Fourthly, acute exposure to physiological level of 17β-estradiol enhanced β_2-adrenoceptor-mediated relaxation. Finally, ovariectomy and chronic 17β-estradiol altered the vascular reactivity in mainly endothelium-denuded arteries. These data suggest that chronic treatment of 17β-estradiol may modulate the properties of smooth muscle but not of endothelial cells.

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